TOXICOLOGICAL SUMMARY AND SUGGESTED ACTION LEVELS TO REDUCE POTENTIAL ADVERSE HEALTH EFFECTS OF SIX CYANOTOXINS

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Integrated Risk Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

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DRAFT FINAL REPORT

June 2009

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Executive Summary

Purpose: Worldwide, several species of cyanobacteria produce cyanotoxins causing human illnesses and killing pets or livestock. Cyanobacteria bloom in California surface water bodies. These blooms have caused public alarm but local health officials lacked a health basis for actions such as posting warning signs. The California State Water Resources Control Board (SWRCB) contracted with the Office of Environmental Health Hazard Assessment (OEHHA) to provide risk assessment support on cyanobacterial toxins. OEHHA used risk assessment to compute the risk-based concentrations. Risk assessment has two parts: toxicity assessment and exposure assessment.

Toxicity assessments are conducted on specific chemicals. There is sufficient toxicological information for six cyanotoxins: anatoxin-a, cylindrospermopsin and the four microcystins; LA, LR, RR, and YR. OEHHA computed a dose above which adverse health effects could occur called a Reference Dose (RfD). The RfDs are based on the published literature for each chemical based on serious health effects like liver damage. RfDs differ for acute one-time and sub chronic multi-day exposures. OEHHA computed separate RfDs for humans, pets, and livestock.

Exposure assessments quantify the dose of chemicals people or animals take in assuming different scenarios. People can inadvertently ingest contaminated water during recreational uses of surface water such as swimming, boating, and waterskiing. In addition, these recreational users can inhale toxins that are aerosolized, and can absorb toxins through their skin. People fishing in a contaminated area may later be exposed to cyanotoxins when they ingest the contaminated fish or shellfish they caught. Equations relate cyanotoxin concentrations in water or fish to doses people ingest, inhale and absorb through the skin for each of these scenarios. Pets can ingest cyanobacterial scum or drink contaminated water.

Action Levels: OEHHA computed health-based water concentration levels for people, pets and livestock. Health based concentrations in sport fish and shellfish were also computed. The exposure equations and RfDs described above were used to calculate suggested action levels. The following table shows the results of these computations.

Action levels for selected scenarios

	Microcystins[1]	Anatoxin-a	Cylindro-spermopsin	Media (units)
Human recreational uses[2]	0.7	50	4	Water (µg/L)
Human fish consumption[3]	13	1100	66	Fish (ng/g)
Subchronic action level, dog[4]	1	40	4	Water (µg/L)
Acute action level, dog[5]	50	40	50	Water (µg/L)
Acute action level cattle	200	100	200	Water (µg/L)
Subchronic action level, cattle	3	100	10	Water (µg/L)

[1] Microcystins LA, LR, RR, and YR all had the same RfD so the action levels are the same.
[2] The most highly exposed of all the recreational users were 7- to-10-year-old swimmers. Boaters and water-skiers are less exposed and therefore protected by these action levels.
[3] For consumption of self-caught fish and shellfish.
[4] Subacute refers to exposures over multiple days.
[5] Acute refers to exposures in a single day

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Preface

This document is a deliverable item under a contract between the State Water Resources Control Board (SWRCB) and the Office of Environmental Health Hazard Assessment (OEHHA). OEHHA and SWRCB are members of the California Environmental Protection Agency (Cal/EPA). The SWRCB is charged with protecting California's waters. OEHHA scientists have expertise in toxicological evaluations. OEHHA frequently provide support for human and nonhuman risk assessment issues.

Introduction

Some species of cyanobacteria (also called blue-green algae) produce toxins, collectively referred to as cyanotoxins. Several cyanotoxins are extremely toxic to laboratory animals and have poisoned people. Cyanobacteria and cyanotoxins are found in lakes, reservoirs, rivers and estuaries throughout the world, including California, although the amount can vary drastically between water bodies and times of the year. People swimming, waterskiing, or boating in these water bodies can be exposed to cyanotoxins. Cyanotoxins may also accumulate in fish that are caught and eaten by people. Finally, pets and livestock have died after drinking water contaminated with cyanotoxins. California public health officials need a basis for decisions regarding recreational and other uses of these water bodies. This report provides a basis for these decisions:

- The report summarizes published toxicological information concerning six cyanotoxins: anatoxin-a, cylindrospermopsin, microcystin LR, microcystin RR, microcystin YR and microcystin LA
- Using this published information, the report establishes reference doses for each of these toxins above which adverse human health effects could occur.
- The report describes methods for estimating exposure during recreational use of water bodies and combines these exposure estimates with the reference dose to estimate water concentration for each toxin that protects recreators.
- Similarly, the report describes methods for estimating exposure to domestic animals and combines these exposure estimates with the acute and subchronic reference doses to estimate water concentrations for each toxin to protect pets and livestock.
- The report provides a literature survey of the effects of cyanotoxins on aquatic ecosystems.

More specifically, in this research effort OEHHA staff have:

1. Identified the health effects that may occur upon exposure to the six cyanotoxins in both humans and domestic animals.
2. Determined dose levels that may result in adverse health effects, for various exposure durations.
3. Identified routes by which exposure may occur under various exposure scenarios.

4. Developed scientifically based health protective "action levels" that may be applied as needed, by local, regional, state or tribal entities throughout California, to reduce (or eliminate) algal toxin exposures.
5. Highlighted any data gaps or areas of further research that may be useful in addressing the challenges identified with this work.

Research Strategy & Results

OEHHA staff searched scientific bibliographic databases on the subject of cyanotoxins. The initial searches identified about 1500 scientific papers on the topic of adverse health effects and exposure to cyanotoxins. Many of these papers were acquired and reviewed. Often a reviewed paper would cite other papers and reports that were also acquired and reviewed. OEHHA staff further examined review articles, guidance documents and various government communications on the same topics. At the end of the project OEHHA staff had identified 2025 publications relevant to the topic.

Additional Resources

While researching this topic, a number of individual experts in the field of cyanobacteria and their toxins were identified. These individuals may be helpful in addressing any number of cyanobacterial-related issues. A Blue Green Algae Work Group including representatives of the State Water Resources Control Board, the Department of Public Health, and the Office of Environmental Health Hazard Assessment has produced a draft document - Cyanobacteria in California Recreational Water Bodies, providing Voluntary Guidance about Harmful Algal Blooms, Their Monitoring, and Public Notification, which is available at:
http://www.waterboards.ca.gov/water_issues/programs/bluegreen_algae/docs/bga_volguidance.pdf

What is Not Addressed

Chemical concentration levels versus cyanobacterial counts

Observing the presence of cyanobacteria is not difficult, so cyanobacteria are often identified in water bodies. However, cyanobacterial counts do not provide adequate information, since it is the toxins and not the cyanobacteria that cause severe toxicity. Unfortunately, the complexity of the relationship between the presence and quantity of cyanobacteria and concentrations of cyanotoxins in the water precludes estimating toxin concentrations from cyanobacterial counts. Cyanobacterial counts can overestimate the risk of cyanotoxin poisoning if cyanobacteria are present but not producing toxin. They can also underestimate the risk of cyanotoxin poisoning because cyanotoxins may persist in the water after a cyanobacterial bloom has subsided. Furthermore, some species of cyanobacteria can produce more than one toxin and the individual toxins can be produced by more than one species of cyanobacteria. Therefore, public health decisions require measured concentrations of these cyanotoxins, not cell counts.

Not all cyanotoxins are considered

Cyanobacteria produce toxins other than the six listed above. There are over 80 similar but distinct microcystins called analogs; only four are addressed in this document. Over the last ten years the number of microcystin identified analogs has grown significantly and there may be analogs yet to be identified. Criteria can be developed for chemicals with quality toxicological studies. Toxicological studies were found to support the development of toxicity criteria for only six cyanotoxins. Therefore, this document excludes many cyanotoxins. Fortunately, in dealing with chemical analogs, scientists look for those with the highest toxicity. The six chemicals identified in this report are among the most toxic cyanotoxins known.

Nonspecific symptoms not considered

Only health effects such as liver damage or neurological toxicity that can be unequivocally linked with specific doses of cyanotoxin are considered. This excludes a number of symptoms including skin lesions, blisters, vomiting, headache, pneumonia, fever, sore throat, blistered mouth, ear and eye irritation, abdominal pain, visual disturbances, diarrhea, and flu-like symptoms.

Drinking water exposure not assessed

The action levels suggested in this document are not intended to apply to treated or untreated water that is intended for drinking, which may be consumed in much larger quantities than incidental ingestion during recreation. There is a separate process by which drinking water risks are assessed and mitigated.

Cyanotoxins and Potential Health Effects

This section presents a) the chemical structure of the six cyanotoxins that are the subject of this document, b) the occurrence of these chemicals in California, and c) a summary of human poisonings as well as effects on non-human species.

What are Cyanotoxins?

Cyanotoxins are chemicals produced by cyanobacteria that can induce toxic effects. There are an enormous number of cyanobacterial species that live in marine, fresh or brackish waters. Cyanobacteria may or may not produce one or more toxins. The conditions that favor toxin production are not well understood.

Microcystins

Microcystins are the most numerous of the cyanotoxins. There are over 80 analogs of these cyclic peptides containing seven amino acids synthesized by multiple genera of cyanobacteria, most commonly *Microcystis*. Figure 1 shows the general structure shared by all microcystins with variable portions shown as X, Z, R^1 and R^2.

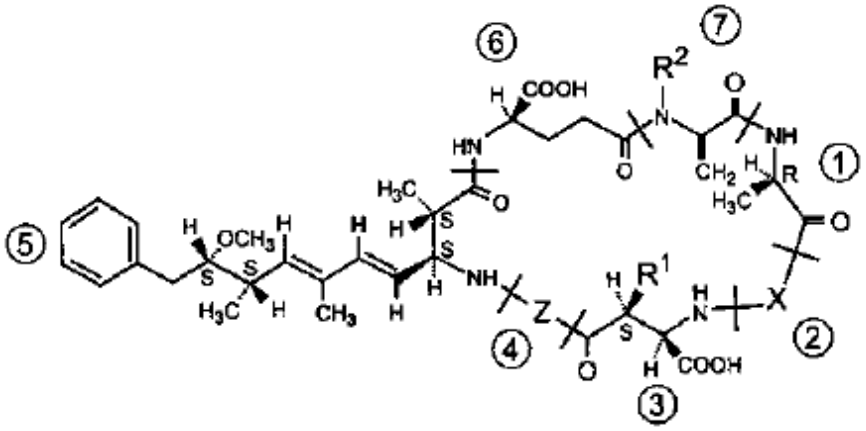


Figure 1: General structure of microcystins

The four microcystins addressed in this document have different amino acids in the X and Z positions in the figure above, but are otherwise identical (both R1 and R2 are methyl groups). Microcystins are named using the one letter abbreviation for the amino acids substituted at the X and Z positions, respectively. Table 1 shows the amino acids that would appear in the structure above for the named microcystins.

Table 1: Composition of Microcystin Congeners

Name	X-position Amino Acid	Z-position Amino Acid	Molecular Weight
Microcystin LA	Leucine	Alanine	910.06
Microcystin YR	Tyrosine	Arginine	1045.19
Microcystin RR	Arginine	Arginine	1038.2
Microcystin LR	Leucine	Arginine	995.17

Cylindrospermopsin

Cylindrospermopsin is a single chemical with the structure illustrated in Figure 2. Cylindrospermopsin is produced by *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Rhaphidiopsis curvata*, *Anabaena lapponica* and *Anabaena bergii*. These species are found in Australia, New Zealand, Europe, Asia and the Americas.

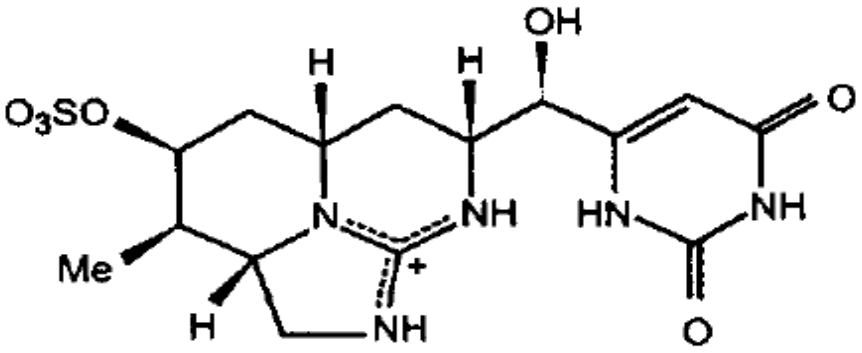


Figure 2: Structure of cylindrospermopsin

Anatoxin

Anatoxin-a has a chemical structure shown in Figure 3. It is produced by species of several cyanobacterial genera including *Anabaena, Planktothrix (Oscillatoria), Aphanizomenon* and others.

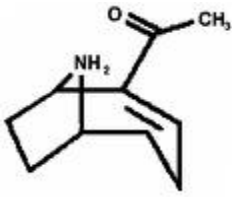


Figure 3. Structure of anatoxin-a

Persistence of Cyanotoxins

Microcystins

Microcystins are extremely stable and resist common chemical breakdown such as hydrolysis or oxidation under conditions found in most natural water bodies. They are

even stable in boiling water [1 , 2]. Tsuji et al. [3] found microcystins to break down slowly in full sunlight especially when water-soluble pigments are present.

The cyclic peptide microcystins are not susceptible to eukaryotic protease and many bacterial proteases. However, there are proteases in some naturally occurring bacteria that are capable of degrading microcystins [4-7]. Because these microcystin-proteases are not everywhere, microcystins may persist for months or even years once released into cooler dark natural water bodies lacking the bacteria that can degrade them.

Cylindrospermopsin

Like microcystins, cylindrospermopsin can be boiled for 15 minutes with no effect and it is relatively stable in the dark. However, it will slowly break down ($t_{1/2}$ = 9hrs) at temperatures >50 °C). Pure cylindrospermopsin is relatively stable in sunlight, but the presence of cell pigments leads to rapid photolysis. Photolysis can break down more than 90 per cent of the cylindrospermopsin within 2-3 days [8].

Information on microbial degradation of cylindrospermopsin could not be found.

Anatoxin-a

In the dark, anatoxin-a is relatively stable. But in pure solution, it is rapidly degraded by sunlight (photolysis) which is accelerated by alkaline conditions [9]. The half-life was found to be approximately 14 days under normal light conditions at pH 8 or pH 10 with an initial concentration of 10 µg/L [10].

Anatoxin-a is also degraded by bacteria associated with cyanobacterial filaments [11, 12], but was not degraded in cyanobacterial filaments free of contaminating bacteria [13]. A five-day half-life was measured in samples of lake sediment and natural bacteria in the laboratory [10].

Cyanotoxins Are Found in California

Only chemical analysis can determine if specific cyanotoxins are present in a water sample. This expensive process is rarely done unless motivated by some concern for human or ecological health. More often, cyanobacteria known to produce cyanotoxins are identified in a water body. But not all blooms in California have been observed and reported. Therefore, the following incidents do not represent all of the surface water in which cyanotoxins could likely be found.

Microcystins have been measured in the Salton Sea of Imperial County [14]. The Iron Gate reservoir and Copco Lake have been sampled numerous times over the past few summers and microcystins have been detected - sometimes at high concentration [15, 16]. Along the Eel River, dogs died in 2002 and 2004; anatoxin-a was found in the

stomach contents of two of the dogs [17]. Anatoxin-a has also been identified in the Eel River [18]. Four lakes in southern California (Lake Mathews, Lake Skinner, Diamond Valley Lake, and Lake Perris) were found to have measurable levels of microcystins [19]. Microcystins have been measured in the Delta region east of San Francisco Bay and up into the Sacramento and San Joaquin Rivers [20, 21]. Cylindrospermopsin has not been reported to be found in California, and no scientific papers were found in which samples from California water bodies were tested for this toxin. Cylindrospermopsin has been found in Florida [22].

In temperate climates, cyanobacterial blooms are associated with higher water temperature, increased pH, low turbulence and high nutrient inputs, showing a characteristic seasonal pattern [2, 23]. Toxin-producing cyanobacteria have flourished in stagnant water along the Klamath River in August or September [16].

Cyanotoxin Poisonings

Human Poisonings

No Human Deaths from Ingestion

While there have been impacts on human health, no human deaths from ingestion of cyanotoxins have been reported in the scientific literature. In 1999 the World Health Organization convened a panel of international experts and produced what remains the most comprehensive review of the field. “In comparing the available indications of hazards from cyanotoxins with other water-related health hazards, it is conspicuous that cyanotoxins have caused numerous fatal poisonings of livestock and wildlife, but no human fatalities due to oral uptake have been documented” [2]. Although there is a single newspaper account of a human fatality [24], the relationship of this death to cyanotoxins has been seriously questioned. Anatoxin-a was initially identified in the stools, blood, and other fluids from a boy but that was later determined to be a spurious result. Dr. Carmichael, an international expert on cyanotoxins doubts the causal role of anatoxin because anatoxin-a induces symptoms almost immediately after the toxin is absorbed from the gut - not 48 hours later as in the case in question. A year later, at an international scientific meeting, Dr. Carmichael explained that the analytical method he used to measure anatoxin-a in the biological samples can misidentify phenylalanine, a common amino acid, as anatoxin-a [25].

Human Deaths after Intravenous Exposure

In February of 1996, following routine dialysis, 116 of 131 patients in Caruaru, Brazil experienced visual disturbances, nausea, vomiting, and muscle weakness. One hundred then developed acute liver failure and 52 eventually died from symptoms now called “Caruaru Syndrome” [26]. Cyanotoxins in reservoir water used in the dialysis caused this syndrome [27]. Cylindrospermopsin and microcystins were found in the water; microcystins were also found in the blood and livers of the patients.

Nonfatal Health Effects from Cyanotoxin Exposure

There are numerous reports of a variety of health effects after exposure to cyanotoxins in either drinking water or as a result of swimming in water in which cyanobacteria were present. Cylindrospermopsin in drinking water poisoned several people in Australia. No one died, but liver enlargement, kidney damage, profuse bloody diarrhea, and fever were reported. Many of the exposed patients required intravenous intervention to maintain electrolytes [28].

Animal Poisonings

The majority of reported cyanotoxin poisonings have occurred in domestic animals that drink from freshwater bodies containing cyanobacterial blooms [see reviews by 29, 30-32]. Unfortunately, some animals appear to be attracted to cyanobacteria in water and dried crusts [reviewed by 31]. Livestock and dogs have been observed to drink cyanobacteria-infested water while clean water was plainly accessible, and to avidly consume crust and mats [33-36]. Lopez-Rodas and Costas [34] found that mice showed a clear preference for *Microcystis aeruginosa* scum (1,000 and 15,000 cells/ml) over clean drinking water. These mice did not prefer non-cyanobacterial phytoplankton over clean drinking water and did not differentiate between toxic and non-toxic strains of the cyanobacteria. These observations and experiments indicate that at least some animals preferentially consume cyanobacteria.

A brief overview of cyanotoxin poisonings in livestock and pet dogs is provided below. Early reports typically include the type and numbers of animals affected, the species of cyanobacteria present and, when possible, symptoms prior to death and time to death. In recent decades, a greater effort has been made to obtain a definitive diagnosis of cyanotoxin poisoning when investigating animal mortalities. A common approach includes analysis of water (including cyanobacterial cells), stomach contents and key organs for suspected cyanotoxin(s). Additionally, several papers have detailed diagnostic approaches that utilize histopathologic and biochemical analyses to provide a stronger diagnosis [18, 35-39].

Livestock

Thousands of livestock fatalities have been linked to the ingestion of cyanobacteria [reviewed by 29, 31]. Several distinct cyanotoxins have been implicated in the poisonings including microcystins [e.g., 37, 40] anatoxin-a [41] and cylindrospermopsin [42]. Animal deaths resulting from cyanotoxin poisoning have been reported on every inhabited continent. The most frequent and severe events have occurred in Australia, where 10,000 livestock died following a large bloom of *Anabaena circinalis* in the Darling River [reviewed by 29]. *Microcystis* spp. and *Anabaena* spp. are most commonly reported in

conjunction with livestock poisonings. *Cylindrospermopsis* spp. poisonings have been the least reported, mainly occuring in Australia [42-44].

In North America, cyanotoxin poisonings in cattle, horses, sheep, pigs, chickens and turkeys have been reported. Livestock poisonings have been linked to blooms of *Microcystis* sp. in Oklahoma [45], Mississippi [46], Georgia [47], Wisconsin [48], Michigan [49], Colorado [37] and Saskatchewan, Canada [50, 51]. Livestock poisonings linked to *Anabaena* spp. blooms have been reported in Oklahoma [45], Kentucky [52], Illinois [53], Iowa [reviewed by 31] and Alberta and Saskatchewan in western Canada [41, 50, 51]. Most of the poisonings were fatal and were associated with visible scum of cyanobacteria.

Livestock poisonings have even occurred under environmental conditions considered unfavorable to cyanobacteria blooms such as cold temperatures and low nutrient levels. Over 100 cattle deaths have been linked to microcystins in high alpine lakes of Switzerland with very low temperature and nutrient levels [40]. In these cases, *Planktothrix (Oscillatoria)* sp., a benthic cyanobacterium, produced the microcystin. Similarly, a *Microcystis* sp. bloom that occurred in a Michigan pond during cold weather was determined to be the cause of poisoning in four yearling Holstein heifers (one survived) [49]. In another case, a *Microcystis* sp. bloom in a Georgia pond during mid-November was linked to the deaths of 4 cattle [47]. Temperatures were cold but an adjacent field had just received high nitrogen fertilizer, which likely supported the bloom. Cattle losses would have been greater but for rapid diagnosis and removal of pond access. These cases demonstrate that toxic blooms occur in atypical environments.

Dogs

Numerous poisonings in dogs have been ascribed to the ingestion of cyanobacteria around the world [29, 31, 32]. Dog deaths have been reported following the consumption of cyanobacteria that produce anatoxin-a or microcystins in Europe, Africa, New Zealand, Canada and the United States. In recent decades, diagnoses of the specific cyanotoxin responsible for dog poisonings have frequently been reported. Anatoxin-a poisonings in dogs have resulted from ingestion of benthic *Planktothrix* spp. [18, 33, 54-57] and *Phormidium favosum* [58, 59]. Microcystin poisoning following consumption of *M. aeruginosa* has led to several dog mortalities [60, 61]. In California, dogs have died from anatoxin-a poisonings after consuming benthic *Planktothrix* sp. in the Eel River [18] and from microcystin poisonings after consuming *M. aeruginosa* from an undisclosed location [61].

Health-Based Criteria for Cyanotoxins

As described above, cyanotoxins have adversely affected the health of people and animals and they are found in surface water bodies in California (although the extent of their distribution is not completely defined). Some of these water bodies are used for recreation (swimming, waterskiing, and fishing) that could result in human exposures. Furthermore, livestock and pets may drink contaminated surface water. Public health officials need a basis to prevent or warn of exposures to toxic chemicals that may lead to

adverse health effects. To meet that need, OEHHA has developed health-based surface water concentrations for the six cyanotoxins as the basis for decisions to protect public health and the health of pets and livestock.

There are two parts to determining these health-based surface water action levels for contaminated water bodies:

1) **Toxicity Assessment** is an analysis of amounts or dosages of a chemical taken in by a person or animal that cause adverse health effects.
2) **Exposure Assessment** is a process for estimating how much of the chemical will be taken into the body of a person or animal. This depends on the amount or concentration of the chemical in the environment, and the assumed exposure scenarios, such as drinking the water, or recreational use of the water such as swimming, water-skiing, fishing, etc. Exposure assessment involves the consideration of potential pathways and routes of exposure for each given exposure scenario. In the case of cyanotoxins the environmental exposure medium of primary concern is water.

Toxicity Assessment of Cyanotoxins

Toxic effects occur when an organ in the body, such as the liver, kidney, or lungs, does not perform its function because of the effects of a toxin. For toxic effects other than carcinogenesis, which involves changes in DNA, there is generally considered to be a "threshold dose" that can be tolerated without toxic effects. The concept of a threshold dose applies to all of the chemicals considered in this document.

Toxic chemicals interact with components of cells, leading to cell death or disruption of vital cellular function(s). Thresholds exist because the body has mechanisms to prevent harm from many outside chemicals and because of biological redundancy. Because there are many components in each cell and many identical cells, there is a dose of chemical that can be tolerated without inducing a toxic effect. The concept of a tolerated dose is the basis of most health-based regulatory concentration limits for non-carcinogenic effects. This maximally tolerated dose is the maximum dose to which people can safely be exposed. It has been given different names but the term reference dose (RfD) that is used by U.S. EPA is used here. RfDs can be developed for varying exposure durations: acute (<24 hrs), short-term (up to 30 days), subchronic (up to 10 percent of a person's lifetime) and chronic (more than 10 percent of a person's lifetime) exposure durations.

The goal of the toxicity assessment is to estimate an RfD for each of the chemicals. The RfD represents the maximum dose to which people could be exposed without significant risk of adverse health effects. Since there was no direct scientific information on the maximum cyanotoxin dose that would not cause a toxic effect in humans, studies in laboratory animals (mostly rats and mice) were relied on to estimate the RfD. There are three steps in estimating that dose:

- First, identify the best study that provides quantitative information.
- Second, determine a dose that does not cause adverse health effects.
- Third, combine that dose with appropriate uncertainty factors.

For the purposes of this document, appropriate studies are those in which animals were exposed orally to clearly defined doses of pure chemical and then examined for the most sensitive toxicological response for each chemical (i.e. the effect that occurs at the lowest dose). The best studies also have multiple doses with toxicological responses increasing with dose and no toxicological response at the lowest dose, so the area of the "threshold" is defined.

The duration of exposure in animal studies helps dictate the human exposure durations to which the RfD derived from that study can be applied.

Health-Based Criteria for Four Microcystins

Existing Health-Based Criteria

The World Health Organization (WHO) has developed a Tolerable Daily Intake (TDI, equivalent to EPA's RfD) for microcystin-LR of 4 X 10^{-5} (0.00004) milligrams per kilogram body weight per day (mg/kg-d). WHO [2] considered the ability of microcystins to promote liver tumors, but the International Agency for Research on Cancer found the evidence for microcystins to cause cancer in humans inadequate. Consequently, WHO based the TDI on a non-cancer endpoint, liver toxicity in mice [62]. In water containing cyanobacterial cells, this guideline value should be applied to the total cell-bound and extracellular microcystins [2].

Based on their TDI, WHO developed a drinking water concentration criterion (Equation 1). It includes an exposure assessment that relates a concentration in water to a dose taken into the body. It assumes that a 60 kg person drinks two liters of water each day and that 80 percent of the two liters is from a contaminated source.

$$\frac{TDI \times BW}{IR \times RSC} = DWC \qquad \text{Eq. 1}$$

where,

TDI= Tolerated Daily Intake, 0.00004 mg/kg-d
BW= Body Weight, 60 kg
IR= Intake Rate, 2 L/d
RSC= Relative Source Contribution, 0.80
DWC= Drinking Water Concentration Criterion, 0.0015 mg/L or 1.5µg/L

The most recent publication [63, 64] cites the 1998 provisional guideline of 1 µg/L based on the equation above and rounded to one significant digit (rounding down to be health-protective).

WHO also categorizes swimming risk levels as mild, moderate, high or very high based on the water concentration of microcystins, as shown in Table 2. These water concentrations are related to the likelihood that a 60 kg swimmer ingesting 100 ml of water would exceed the TDI. However, as previously stated, OEHHA does not agree that concentration of microcystins can be estimated by algal counts or by observing scum.

Table 2. World Health Organization Guidelines

Risk Level	Microcystin (µg/L)	Estimated algal equivalent
Mild	2	No scum low algae count
Moderate	20	No or little scum algae dispersed in top 4 meters of water
High	2,000	100-fold accumulation to high risk level scum in top 4 cm
Very high	20,000	1,000-fold accumulation to very high risk level shore scum if wind sweeps scum from 100 m into 10 m

A survey of the government regulations regarding cyanotoxins in18 countries indicates that if they have regulations, they all rely on the WHO criteria for microcystins [65, 66]. No regulatory criteria were found for anatoxin-a or cylindrospermopsin.

Toxicology of Microcystins

The USEPA publishes toxicological reviews of specific chemicals. A primary objective of these reviews is to determine the RfD. In November of 2006, USEPA released a draft toxicological review for microcystins [67], which is still under revision and subject to change. OEHHA used this review and the references it contained, along with other references, in preparing the following discussion of the toxicity of the four microcystins. The effects of both purified microcystins and unpurified cyanobacterial extracts on animals have been studied. In the unpurified cyanobacterial extracts, the microcystin isomers are sometimes inferred by the species of cyanobacteria from which the extracts were prepared.

Microcystins in general are liver toxins; humans and other species poisoned by microcystins show clear hepatotoxicity [27]. Most of the understanding about the toxicity of microcystins is based on mice and rats receiving intraperitoneal (ip) injections (i.e. directly into the abdominal cavity) of microcystin-LR. Early manifestations of liver damage include an increase in liver enzymes released into serum and increased liver weight as blood fills the liver. Liver damage and cell death can also be seen microscopically. Liver changes have been observed in mice as soon as 20 minutes following injection of a lethal dose of microcystin-LR. By an hour post-dosing, the liver cells are dying, disconnecting from one another and disrupting the normal architecture of the liver [68, 69]. Microcystins can induce death in a few hours. Two mice given oral doses of 16.8 and 20 mg/kg were dead within 160 minutes [70].

Cells die in distinctly different ways. Hepatocytes from animals poisoned with microcystins appear to die by apoptosis [71]. Apoptosis, the scientific term for programmed cell death or cell suicide, has been intensively studied in developmental biology. Cells undergoing apoptosis disappear in a characteristic fashion, cannibalizing their own cellular organelles [72]. Microcystins have been used to investigate the biochemical pathway initiating apoptosis [73]. Apoptosis involves a series of proteins each chemically transforming the next. Adding phosphate to, or removing it from, a protein is a common step in a biochemical pathway. Protein phosphatases remove phosphates from proteins. Microcystins inhibit a certain class of protein phosphatases. This inhibition and the subsequent buildup of phosphorylated proteins are believed to be a mechanism by which microcystins destroy livers. There is some evidence that microcystin-LR increases other proteins in pathways leading to apoptosis but this is not as extensively studied as is the inhibition of phosphatases [74].

While the most extensive toxicological information is available for the microcystin-LR congener, the LA, RR and YR congeners appear to have similar toxicological effects: these congeners induce histological changes in rodent liver similar to microcystin-LR and have been shown to inhibit the same phosphatases [75]. Therefore, the toxicity criteria computed for microcystin-LR will be used for microcystins LA, RR and YR. This mechanism of toxicity may also apply to other microcystins, but that has not been confirmed.

Microcystins and Cancer

The International Agency for Research on Cancer convened a panel of international experts to evaluate the carcinogenic potential of both microcystin-producing cyanobacteria (*Microcystis*) and purified microcystin-LR. A preliminary report of this committee is available [76]. The panel reviewed epidemiology studies showing increases in liver and colon cancer in people who drank surface water that likely contained *Microcystis* (as well as other chemicals) compared with those who consumed well water [77]. The committee found these studies interesting, but "*Microcystis* extracts are not classifiable as to their carcinogenicity to humans." Furthermore, the committee reviewed studies in rats and mice exposed to *Microcystis* extracts and microcystin-LR. They concluded there was "inadequate evidence" that either *Microcystis* extracts or microcystin-LR causes cancer in laboratory animal. Surprisingly they concluded that "There is inadequate evidence in humans for carcinogenicity of microcystin-LR," but the overall evaluation was "Microcystin-LR is possibly carcinogenic to humans." in the draft document. The committee felt there was strong evidence supporting a plausible tumor promoter mechanism [77].

There have been no definitive studies published on the ability of microcystins to cause cancer in humans or animals since the committee met in 2006. However, the National Toxicology Program (NTP, a division of the U.S. National Institutes of Health), plans to conduct a Carcinogenicity/Toxicity test in rats by intravenous exposure [78].

The mechanisms of carcinogenesis can be divided into genotoxic and non-genotoxic. Genotoxic carcinogens can cause a permanent mutation (change) in the DNA structure of a cell, leading to uncontrolled growth of cells, i.e. cancer. Non-genotoxic carcinogens do not need to cause mutations in DNA to increase cancer. One kind of non-genotoxic carcinogen is called a tumor promoter. A specific protocol is used to identify tumor promotion. Animals are first exposed to a genotoxic carcinogen called an initiator. These initiated animals are then exposed to varying doses of a second chemical. If that chemical increases the number or size of tumors in a dose-dependent fashion, the chemical is considered to have tumor promoter properties.

Falconer [79, 80] reported in a letter to the editor that drinking water administration of *Microcystis* extracts to mice increases both the number and weight of skin tumors in mice treated topically with the carcinogen dimethylbenzanthracene. Rats treated with diethylnitrosamine develop liver tumors that are preceded by pre-cancerous foci of liver cells that express a number of enzymes atypical for liver. In a short-term liver tumor promoter assay, *Microcystis* extracts increased the number of liver foci in diethylnitrosamine-treated rats in a dose-dependent fashion [81]. Interestingly, *Microcystis* extracts decreased duodenal tumors in mice in the only study in which oral dose levels were reported [82]. OEHHA's review of the literature finds that there is evidence suggesting a potential for microcystin-LR to promote rodent liver tumors induced by a genotoxic carcinogen. However, there are no dose-response studies available that would allow computation of a criterion based on tumor promotion. Therefore, OEHHA's RfD is based on liver toxicity.

Reference Dose in Humans

Two potential studies are available on which to base a short-term RfD: The Fawell mouse study used in determining the WHO TDI [2] and the Heinze rat study [83]. The Heinze study was not published in time to be reviewed for the WHO report [2]. Rats showed signs of toxicity at lower doses than the mice, so the Heinz study was selected as the basis of the RfD. Heinze [83] exposed two groups of ten rats each to microcystin-LR-laced drinking water for 28 days. A control group was given plain drinking water. The rats were weighed weekly and the concentrations in their drinking water were adjusted so that the low dose got 50 µg/kg-d and the high dose group got 150 µg/kg-d. On day 28, the rats were sacrificed. Organ and body weights were recorded, blood and serum clinical chemistry parameters were measured and histological sections of liver and kidney were examined microscopically. The incidence of microscopic liver lesions (0 of 10 at 0 µg/kg-d, 6 of 10 at 50 µg/kg-d and 9 of 10 at 150 µg/kg-d) was selected as the toxicity endpoint for both the short-term RfD calculations because this endpoint showed a clear dose-response trend. Other candidate endpoints either did not show a clear dose-response trend (increased serum levels of lactate dehydrogenase and alkaline phosphatase or were less sensitive (liver-to-body weight ratio).

The incidence of microscopic liver lesions was input into the EPA benchmark dose software (version 1.3.2). This software fits various mathematical models to the dose-

response data to estimate the dose associated with a 10% response rate (BMD) and a 95% lower confidence limit on the BMD (BMDL). The log-probit fit of the data was determined to be the best fitting model and this resulted in BMDL estimate of 0.00638 mg/kg-d, rounded to 0.006 mg/kg-d. Dividing this benchmark dose by a cumulative uncertainty factor (UF) of 1000 resulted in an RfD of 6×10^{-6} mg/kg-d. The cumulative UF included

- a UF of 10 because the average human could be as much as 10 times more sensitive to the toxic effect of the chemicals than the laboratory animals that were tested,
- a UF of 10 because the most sensitive human could be as much as 10 times more sensitive to the toxic effects of these chemicals than the average human
- a UF of 10 because complete toxicology profiles are not available for these chemicals particularly with regard to cancer and effects in children.

The same value and computation were used for both the short term and subchronic RfDs. Therefore, this RfD could apply to daily exposures ranging from one day to seven years (10 percent of 70 years).

Two studies in mice are available to determine a chronic RfD. A gavage study by Ito et al. [84] did not provide enough information to determine an average daily dose. An 18-month drinking water study by Ueno et al. [85] is suitable. The study used only one dose level (a concentration of microcystin-LR that resulted in a dose of 3 µg/kg-d) and a control group. There was no difference in any of the parameters measured in the treated mice compared to the control mice. Therefore, 3 µg/kg-d was determined to be a No Observable Adverse Effect Level (NOAEL). Their cumulative uncertainty factor of 1000 (similar to that used in the subchronic RfD) resulted in a chronic RfD of 3×10^{-6} mg/kg-d, one-half of the short-term and subchronic RfD value.

These computations and reasoning are the same as those described in the EPA draft document describing subchronic and chronic RfDs for microcystins.

Acute Reference Dose in Domestic Animals (based on lethality)

Jackson et al. [86] exposed fifteen sheep to varying amounts of lyophilized *Microcystis aeruginosa* collected from a natural bloom. A single bolus of cyanobacteria was introduced directly into the rumen of the sheep, simulating ingestion exposure. They tested a dose range of 730 to 1,840 mg dry algae per kg body weight. One of 2 sheep given 1040 mg lyophilized *M. aeruginosa/*kg (and all sheep receiving higher dosages) died. The highest oral non-lethal dose was 1010 mg lyophilized *M. aeruginosa* per kg body weight. This dosage of lyophilized cells is equivalent to approximately 3.7 mg microcystin per kg body weight (see appendix IV for this conversion). This dose was divided by a cumulative uncertainty factor (UF) of 100, yielding the acute RfD of 3.7×10^{-2} mg /kg-d for microcystin in domestic animals. The cumulative UF included 10 for

interspecies variation and 10 for insufficient toxicology data and severity of the endpoint. This RfD is intended to protect the average animal rather than the most sensitive, so an intraspecies UF was not applied. The cumulative UF is more conservative than that used in developing the subchronic RfD, discussed next, because of the severity of the endpoint.

Subchronic Reference Dose in Domestic Animals

As described above, Heinze [83] exposed rats to microcystin-LR in drinking water at doses of 0, 50 and 150 µg/kg-d. Following 28 days of exposure, microscopic liver lesions were observed in a clear dose-response trend. The dose associated with a 10% response rate (with 95% lower confidence limit) for microscopic liver lesions was 0.006 mg/kg-d (EPA benchmark dose software v. 1.3.2). This benchmark dose is a reasonable basis for a subchronic RfD for domestic animals. Typically, risk assessments for non-human species do not employ the same level of conservatism as do human health risk assessments. Therefore, we applied a combined UF of 10 to cover the uncertainty in extrapolating from mice to cattle and dogs, and the uncertainty due to incomplete toxicology profiles. Dividing 0.006 mg/kg-d by 10 yielded a subchronic RfD of 6×10^{-4} mg/kg-d. This RfD is intended to protect the average animal rather than the most sensitive, so an intraspecies UF was not applied.

Health-based Criteria for Cylindrospermopsin

Toxicology of Cylindrospermopsin

Animal studies have consistently shown severe liver and kidney damage due to cylindrospermopsin. In contrast to microcystins, the mechanism by which this toxin causes organ damage is unclear. There are three alternative hypotheses: One hypothesis involves the ability of cylindrospermopsin to inhibit protein synthesis [87, 88]. The second hypothesis is that cylindrospermopsin interferes with the ability of mitochondria to produce ATP (adenosine triphosphate) [89]. The third hypothesis involves depletion of glutathione, a chemical produced by the liver and known to protect the liver from reactive chemicals [90, 91].

Kidney structure and function are also affected by cylindrospermopsin. Kidneys are essential because they filter metabolic wastes from the blood and flush them out in the urine. When kidneys are damaged, the metabolic wastes can build up in the blood and lead to death. Kidney to body weight ratio was increased in mice treated with cylindrospermopsin [92]. This generally indicates that there is some renal pathology. There was also histological evidence of damage to the kidney of treated mice. All of this indicates that cylindrospermopsin has a toxic effect on the kidney. In addition, thymus and spleen have been identified as targets of cylindrospermopsin [88, 93]. However, these tissues are affected at higher doses than the kidney and liver. Mice given 30 – 40 mg cylindrospermopsin/kg die within 24 hours [94, 95].

Cylindrospermopsin and Cancer

There are no cancer studies on cylindrospermopsin in animals or epidemiological evidence for carcinogenesis in humans. There are few studies on the genotoxicity of cylindrospermopsin, but there is some evidence that cylindrospermopsin interacts with DNA or causes mutations [96-98] and some weakly positive results in an initiation assay [99, 100]. Given the minimal number of studies on cancer and genotoxicity, OEHHA concurs with the USEPA assessment that there is "inadequate information to assess carcinogenic potential." [99]

Reference Dose in Humans

Cylindrospermopsin's effect on animals has been studied with both purified cylindrospermopsin and extracts of the cyanobacterium *Cylindrospermopsis raciborskii*. While extract studies are interesting, studies on purified toxin are preferred when available because they avoid the effects of contaminating substances. OEHHA adopts the draft subchronic RfD found in USEPA's "Draft Toxicological Reviews of Cyanobacterial Toxins: cylindrospermopsin" released in November of 2006 [99]. This document, which is still under revision by EPA and subject to change, proposes a subchronic RfD based on increased kidney to body weight ratios in mice as shown by Humpage and Falconer [92]. The authors gavaged groups of 10 mice with 0, 30, 60, 120 or 240 µg/kg-d of cylindrospermopsin in water for 11 weeks. A number of parameters were measured, but kidney to body weight ratios were increased at the lowest dose. The Benchmark Dose Software (BMDS, version 1.3.2) fit a mathematical model to the data. The best fit was obtained with a linear model excluding the highest dose group. The 95% lower confidence limit on the benchmark dose was 0.033 mg/kg-d, rounded to 3×10^{-2} mg/kg-d. This value was divided by a cumulative uncertainty factor (UF) of 1000, yielding a subchronic RfD of 3×10^{-5} mg/kg-d.

The cumulative UF included

- a UF of 10 because the average human could be as much as 10 times more sensitive to the toxic effect of the chemicals than the laboratory animals that were tested,
- a UF of 10 because the most sensitive human could be as much as 10 times more sensitive to the toxic effects of these chemicals than the average human
- a UF of 10 because complete toxicology profiles are not available for these chemicals particularly with regard to effects in children..

These computations and reasoning are identical to those described in the EPA draft document describing subchronic RfD for cylindrospermopsin.

Acute Reference Dose in Domestic Animals

No acute oral studies using purified cylindrospermopsin could be found. However, several studies have used cells or cell extracts of *Cylindrospermopsis raciborskii* to investigate acute toxicity following oral dosing. Seawright et al. [93] administered a suspension of freeze-dried cells by gavage to mice. The dose range was equivalent to 4.4 to 8.3 mg cylindrospermopsin/kg. The lowest lethal dose was 4.4 mg/kg. In a similar study in which mice were orally administered 0 - 8 mg cylindrospermopsin/kg as a single dose of sonicated cell extract, the 8 mg/kg dose killed all mice within 48 hours and the 6 mg/kg dose killed two of the four mice exposed within 5 days [98, 101]. No mortality was observed in the remaining dose levels of 0, 1, 2 and 4 mg/kg. Another study reported a higher minimum lethal oral dose of 13.8 mg/kg cylindrospermopsin, as a saline extract of cells, in mice [100]. Based on these studies, the lowest lethal dose of cylindrospermopsin was 4.4 mg/kg and the highest non-lethal dose was 4.0 mg /kg. The latter was divided by a total UF of 100 to yield the acute RfD of 4.0×10^{-2} mg/kg-d for cylindrospermopsin in domestic animals. The cumulative UF included 10 for interspecies variation and 10 for insufficient toxicology data and severity of the endpoint. This RfD is intended to protect the average animal rather than the most sensitive, so an intraspecies UF was not applied. The cumulative UF is more conservative than that used in developing the subchronic RfD, discussed next, because of the severity of the endpoint.

Subchronic Reference Dose in Domestic Animals

As described above, Humpage and Falconer [92] gavaged mice with 0, 30, 60, 120 or 240 µg/kg-d cylindrospermopsin in water for 11 weeks. Increased kidney to body weight ratios in the mice were observed at the lowest dose. The 95% lower confidence limit on the calculated benchmark dose was 0.03 mg/kg-d (Benchmark Dose Software, v. 1.3.2). This dose is a reasonable basis for the subchronic RfD for cylindrospermopsin in domestic animals. We applied a combined UF of 10 to cover the uncertainty in extrapolating from mice to cattle and dogs, and the uncertainty due to incomplete toxicology profiles. Dividing 0.03 by 10 yielded a subchronic RfD of 0.003 or 3×10^{-3} mg/kg-d. This RfD is intended to protect the average animal rather than the most sensitive, so an intraspecies UF was not applied.

Health-based Criteria for Anatoxin-a

Toxicology of Anatoxin-a

Nerves stimulate muscles or other nerves by releasing chemicals called neurotransmitters. Neurotransmitters travel across a thin watery layer called a synapse to either a muscle or another nerve. If enough neurotransmitter binds to receptor proteins on the muscle or post-synaptic nerve, the muscle contracts or the nerve fires. Acetylcholine is a neurotransmitter secreted by many pre-synaptic neurons, especially those connected to muscles. Muscles stop contracting because an enzyme acetylcholinesterase breaks

down acetylcholine so that the receptors return to an empty state. Our rhythmic breathing is controlled by acetylcholine release and its subsequent breakdown. Anatoxin-a can mimic acetylcholine by binding to acetylcholine receptors and stimulating post-synaptic firing. As such it is called an acetylcholine agonist. However, unlike acetylcholine, anatoxin-a is not broken down and so post-synaptic firing does not stop. When an animal's vital muscles like those needed to breathe do not contract rhythmically due to anatoxin-a binding, it stops breathing which is the likely cause of death [102, 103].

Anatoxin-a affects other biological processes both in the brain, and in nerves outside the brain. In rats and mice developmental effects have been observed [104] and neurobehavioral effects have been observed in rats [105-107]. However, anatoxin-a was injected rather than given orally in these developmental and neurobehavioral studies, so they are not useful for establishing a maximum dose for oral exposure.

Anatoxin and Cancer

There are no cancer, genotoxicity or even chronic exposure studies on anatoxin-a. Furthermore, the NTP website does not indicate any plans to test anatoxin-a. OEHHA concurs with the USEPA assessment that there is "inadequate information to assess carcinogenic potential."

Reference Dose in Humans

The best study for a subchronic RfD is one in which three groups of 20 female rats were given either 0, 0.05 or 0.5 mg/kg-d anatoxin-a in their drinking water for seven weeks [104, 108]. No effects were seen at the highest dose, so this is the NOAEL. Applying a 1000-fold uncertainty factor, a subchronic RfD of 5×10^{-4} mg/kg-d is calculated. The cumulative UF was based on the same considerations as that for microcystin. Short-term and sub chronic RfDs are proposed in USEPA's "Draft Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-a" released in the fall of 2006 [109].

The best study for a short-term RfD is one in which groups of 10 male and female mice were gavaged with 0, 0.1, 0.5, and 2.5 mg/kg-d for 28 days [110]. The mice were examined for a wide range of toxicological endpoints both during and at the end of the study. There was no statistically significantly difference between the control group and any of the dosed groups for any of these endpoints. The highest dose, 2.5 mg/kg-d, was identified as the NOAEL. Applying a 1000-fold uncertainty factor (UF) and rounding to one significant digit, a short-term RfD of 0.003 or 3×10^{-3} mg/kg-d was calculated.

The cumulative UF included

- a UF of 10 because the average human could be as much as 10 times more sensitive to the toxic effect of the chemicals than the laboratory animals that were tested,
- a UF of 10 because the most sensitive human could be as much as 10 times more sensitive to the toxic effects of these chemicals than the average human
- a UF of 10 because complete toxicology profiles are not available for these chemicals particularly with regard to effects in children.

These computations and reasoning are identical to those described in the EPA draft document describing subchronic and chronic RfDs for anatoxin.

Acute Reference Dose in Domestic Animals

The work on acute oral toxicity of anatoxin-a is limited. In mice, the oral LD_{50} for purified anatoxin-a has been reported as >5 to 16.2 mg/kg [103, 111] as reported in [2]. Carmichael et al. [102, 111] found calves to be more sensitive to oral doses of *Anabaena flos-aquae* than mice and rats given the same material. The minimum lethal dose (MLD) of a strain of *A. flos-aquae* (NRC-44-1) known to produce anatoxin-a was 525, 1500 and 1800 mg lyophilized cells/kg in calves, rats and mice, respectively. The oral MLD in calves (525 mg cells/kg) is equivalent to 2.3 mg anatoxin-a/kg, since the same *A. flos-aquae* strain was later determined to contain 4.3 µg anatoxin-a/mg lyophilized cells [112]. The anatoxin-a production of strain NRC-44-1 appears to have remained consistent over time since the MLD of lyophilized cells in mice (i.p.) were identical in the two studies [102, 103]. The lyophilized NRC-44-1 cells, however, seem more toxic than purified anatoxin-a since lyophilized NRC-44-1 cells produced an LD_{50} that was less than half of the LD_{50}s of pure toxin and a wild strain of *A. flos-aquae* in mice [103] This suggests that strain NRC-44-1 contains additional toxic elements. Due to its limited size (n=2) and the apparent influence of additional toxins, the calf study was not used to develop an acute RfD for anatoxin-a in domestic animals.

Instead, the anatoxin-a RfD for domestic animals is based on the short-term NOAEL, discussed in the section above [62, 110]. Prior to the 28-day study in mice, Fawell et al. [62, 110] performed a 5-day range-finding study in which groups of mice were orally administered purified anatoxin-a in doses ranging from 1.2 to 12.3 mg/kg-day. The minimum lethal dose was 6.3 mg/kg. Hence, the next lowest dose, 2.5 mg anatoxin-a/kg body weight was the highest non-lethal dose. The fact that a dosage 40 percent of the lowest lethal dose in a 5-day study produced no adverse effects in a 28-day study indicates the very steep dose-response curve of anatoxin-a.

The 2.5 mg/kg dose divided by the UF of 100 yields the short-term RfD of 2.5×10^{-2} mg/kg-d, rounded to 0.03 mg/kg-d, for anatoxin-a in domestic animals. The cumulative UF included 10 for interspecies variation and 10 for insufficient toxicology data and

severity of the endpoint. This RfD is intended to protect the average animal rather than the most sensitive, so an intraspecies UF was not applied. The cumulative UF is more conservative than that used in developing the subchronic RfD, discussed next, because of the severity of the endpoint.

Subchronic Reference Dose in Domestic Animals

As discussed above, the best study for a subchronic RfD is one in which rats were exposed to up to 0.5 mg/kg-d anatoxin-a in drinking water for seven weeks [104, 108]. No effects were seen at the highest dose of 0.5 mg/kg-d, so this is the NOAEL. If a UF of 10 were applied, as was done for cylindrospermopsin and microcystins, the subchronic RfD for domestic animals would be 0.05 mg/kg-d. However, this value is not used because it could result in exposures above the RfD for short-term exposure calculated above (0.03 mg/kg-d). Instead, the short-term RfD of 0.03 mg/kg-d is also applied to subchronic exposures in domestic animals.

Health-Based Water Concentrations for Human Recreational Exposures

To assess potential human exposure to cyanotoxins, various recreational exposure scenarios were considered, including swimming, water-skiing/jet-skiing, fishing, boating, sail boarding, and canoeing. Our analysis (Appendix I) showed that swimmers would have higher exposures than water-skiers, jet-skiers, boaters, sail boarders, and canoers. Therefore, criteria that would protect swimmers would also protect the other recreational users. Exposure from eating fish will be assessed separately, since it is based on concentrations in fish flesh, not on water concentrations.

Inhalation of Cyanotoxins while Boating or Water Skiing

Water skiing, jet skiing or boating can cause cyanotoxins to become aerosols (microscopic liquid or solid particles suspended in air). A skier and boater could inhale these aerosolized cyanotoxins. Cheng [113] measured the concentrations of microcystins in personal air of volunteers recreating on the lake. The concentrations in the water were approximately 1 µg/L microcystins on three sequential days. The results of this study showed that a liter of water contains 700,000 to 800,000 times the amount of the toxins as in a cubic meter of air. (This calculates to 1.3 to 1.4µl aerosolized microcystin/m^3.) Since the assumption used is that a swimmer ingests 50 milliliters/hr, a water-skier would have to inhale at least 35,000 m^3/hr while skiing in order to get a dose equal to the swimmer. This is 17,000 times the inhalation rate of a marathon runner. It is not possible for a water skier to inhale enough aerosol to come close to what a swimmer gets from ingestion (described in the next section). Therefore, a concentration in the water that protects the swimmer should also protect a water skier.

Exposure to Cyanotoxins while Swimming

Cyanotoxins in the water could theoretically enter a swimmer's body along with water that is inadvertently swallowed, by penetrating the skin, and by vaporizing and being inhaled. These three routes of potential exposure are analyzed in Appendix I. That analysis shows that the physical-chemical properties of microcystins and cylindrospermopsin preclude their vaporizing or penetrating the skin to any significant degree, so only ingestion exposure was quantified. On the other hand, vaporization or dermal penetration by anatoxin-a could not be ruled out, so exposure by all three routes was estimated. As described in the section above, aerosols do not contribute significantly when ingestion of water is assumed. Therefore, aerosols are not considered in the swimmer scenario. The results of this exposure analysis are summarized in Table 3:

Health-Based Cyanotoxin Water Concentrations for Swimmers

The ratios in Table 3 were used to determine the water concentration associated with a given dose. As described in Appendix I, the most exposed swimmer is a 7 to 10 year old child. It is assumed that this child is swimming in contaminated water during the summer and early fall when cyanobacterial blooms occur. The Concentration/Dose Ratios for each of the chemicals were multiplied by the corresponding RfD (Section III) to estimate an Action Level, a water concentration above which a child could experience adverse health effects.

Table 3: Cyanotoxin Action Levels for the Swimming Scenario[1]

Chemical	RfD[2]	Concentration/Dose Ratio[3]	Action Level[4]
Units	mg/kg-d	(mg/L) per (mg/kg-d)	µg/L
Microcystins	6×10^{-6}	1.21×10^{2}	0.7
Cylindrospermopsin	3×10^{-5}	1.21×10^{2}	4
Anatoxin-a	5×10^{-4}	1.05×10^{2}	50

[1] Criteria for swimmers also protect other recreational water users
[2] RfDs taken from sections III.B.4, III.C.3 and III.D.3, respectively
[3] See Appendix I
[4] Product of the previous two columns

Ingestion of Cyanotoxins in Fish or Shellfish

The risk involved in eating fish and shellfish containing cyanotoxins has received increased attention in recent years. Ibelings and Chorus [114] provide a comprehensive review of this issue. By far, the bulk of available data pertains to microcystins. Some information is available for cylindrospermopsin while only one study was found on the experimental uptake of anatoxin-a in fish. The major factors contributing to cyanotoxin exposure through fish and shellfish consumption are the concentration of the toxins in these organisms and the amount consumed.

Cyanotoxin accumulation in fish and shellfish.

In their comprehensive review, Ibelings and Chorus [114] concluded that cyanotoxin concentrations in fish are likely to be site-specific and bloom-specific. Fish and shellfish mainly accumulate microcystin and cylindrospermopsin through their diet. Filter-feeding shellfish and planktivorous fish accumulate cyanotoxins by directly ingesting cyanobacteria, especially when thick surface scum is formed. However, even though these organisms are consumed by larger fish, the cyanotoxins do not build up in their tissues because the fish are able to break down much of the ingested cyanotoxins. The extent of cyanotoxin accumulation in biota cannot be predicted based on feeding type or trophic level because microcystin or cylindrospermopsin concentrations at any trophic level depend on several complex interactions including the organism's consumption rate, digestive ability and time since exposure. Time since exposure in fish is an especially important factor for human exposures because microcystin can move from inedible (i.e., liver) to edible (muscle) tissues of fish after the bloom has ceased and fish are no longer being exposed. In fact, cyanotoxins in mussels could be partially retained through the winter because their depuration processes slow down with decreasing temperatures. Although it is clear that microcystin and cylindrospermopsin can be taken up and partially retained by fish and shellfish, site-specific monitoring of cyanotoxins in fish and shellfish during bloom season is necessary to evaluate the risk associated with consumption of those organisms.

Cyanotoxin concentrations found in fish and shellfish

The highest concentrations of both microcystins and cylindrospermopsin are typically found in the liver and gut of the fish, or the hemolymph and hepatopancreas in shellfish [reviewed by 114]. These tissues are not typically eaten (except in mussels and other bivalves) and their removal significantly lowers cyanotoxin exposure in humans. However, elevated concentrations of microcystin and cylindrospermopsin have been measured in edible portions of fish (muscle) and shellfish (muscle or whole). Concentrations of microcystin ($MC\text{-}LR_{eq}$) reported in the literature range from 0.25 - 340 ng/g wet weight (ww) in fish muscle, 5 – 58 ng/g ww in shellfish muscle and 64 – 2,500 ng/g ww in whole mussels [reviewed by 114]. Unpublished data from the California Department of Fish and Game show that mussels and edible portions of fish collected from Klamath River, California and two of its reservoirs contained high levels of total microcystins [115]. Mussels collected from Klamath River in July 2007 contained an average of 554 ± 928 ng MC/g (ww; ± standard deviation). Filets of perch collected from the Klamath reservoirs Copco and Iron Gate in September 2007 had 169 ± 117 and 42 ± 65 ng MC/g ww, respectively.

Up to 205 ng cylindrospermopsin/g ww has been found in prawn flesh. Saker and Eaglesham [116] measured 153 ng/g ww cylindrospermopsin in muscle and 1,290 ng/g ww in hepatopancreas of crayfish from an agricultural pond [converted from dry weight using average percent moisture from 117]. No reports were found of anatoxin-a in fish collected from the field.

Ibelings and Chorus [114] emphasize the importance of understanding the types of fish and shellfish tissues that are consumed locally when assessing the risk associated with consumption. Mussels (and other bivalves) are often eaten whole. Other shellfish are sometimes boiled whole for soups. In some cultures, consumption of whole fish and shellfish is common.

The significance of anatoxin-a uptake in fish to human consumption is currently unclear. When Osswald et al. [118] exposed juvenile carp to *Anabaena* sp. suspensions of 10^5 or 10^7 cells/ml (approximately 12 and 1,170 µg anatoxin-a/L, respectively) for up to 4 days, all fish accumulated < 1% of the available anatoxin-a. In the higher exposure, all fish died within 30 hours and contained 73 ± 71 ng anatoxin-a/g in whole body ww. Carp at the lower exposure survived and accumulated 5 ± 2 ng anatoxin-a/g (ww, whole body). The authors speculate that accumulation would likely be greater in a medium exposure (i.e., between the lower exposure and the unknown lethal threshold), but also point out that the hydrophilicity and instability of anatoxin-a may ultimately result in insignificant accumulation in fish. More work is needed to understand the dynamics of anatoxin-a in the aquatic food web.

Health-Based Cyanotoxin Concentrations in Sport Fish and Shellfish for Consumers

In California, the general fishing population is estimated to consume about 30.5 grams of sport fish and shellfish per day (weighted average of the Santa Monica Bay Seafood Consumption Study; [119], [120]. This consumption rate is equivalent to an uncooked 7.5-ounce fillet each week, which is slightly smaller than the 8-ounce meal size typically used in risk assessments [121]. In order to simplify the action level calculated here, the consumption rate was adjusted to 32 g/day (8-oz/week; uncooked) to reflect a standard meal size.

Action levels for sport fish and shellfish containing microcystins, anatoxin-a and cylindrospermopsin are shown in Table 4. These action levels identify the maximum concentration of cyanotoxins in edible fish and shellfish tissues that a typical consumer (one meal per week) could ingest without exceeding the RfDs. For higher consumption rates, divide the action level by the average number of meals consumed each week. Children are assumed to eat smaller meals (2 – 4 ounces uncooked; see Appendix II). The action levels only apply to the consumption of sport fish and shellfish and do not apply to the consumption of commercial fish and shellfish. Action level calculations are described in Appendix II.

Table 4: Cyanotoxin Action Levels for Sport Fish and Shellfish

Chemical	RfD[1]	Action Level[2]
Units	mg/kg-d	ng/g tissue
Microcystins[3]	6×10^{-6}	13
Cylindrospermopsin	3×10^{-5}	66
Anatoxin-a	5×10^{-4}	1100

[1] RfDs taken from sections III.B.4, III.C.3 and III.D.3, respectively
[2] Based on typical consumption rate of self-caught fish in California (one meal per week) and body weight of 70 kg. See Appendix II for calculations. For higher consumption rates, divide action levels by number of meals per week. Children are assumed to eat smaller meals (2 - 4 ounces uncooked; see Appendix II).
[3] Apply action levels to the sum of all microcystin variants until subchronic toxicities of the other variants are clarified.

Domestic Animal Exposure Assessment

Exposure scenarios in livestock and pet dogs considered here include drinking from water bodies, eating algal-bloom crusts, and swimming (dogs only). For livestock, exposures in dairy and beef cattle were the primary focus.

Livestock

1. Water Ingestion

Acute action levels were calculated to identify the concentration of cyanotoxins in water that represents little or no risk of acutely toxic exposures to cattle (Tables 5 and 6). The cyanotoxin should be measured in total water (cells + water) in order to represent the exposure to cattle. Calculations of water intake rates and related action levels are described in Appendix V.

Table 5: Cyanotoxin RfDs and water action levels[1] for dairy cows

	Microcystin	**Anatoxin-a**	**Cylindrospermopsin**
Water consumption L/kg-d[2]	**0.23**	**0.23**	**0.23**
Acute RfD mg/kg-d[3]	0.04	0.03	0.04
Acute action level µg/L	**200**	**100**	**200**
Subchronic RfD mg/kg-d	0.0006	0.03	0.003
Subchronic action level µg/L	**3**	**100**	**10**

[1] Calculated as: Action level (µg/L) = 1000 x RfD (mg/kg-day) / Intake of contaminated water (L/kg-day). Based on cattle fed dry diet; Action levels for cattle on pasture would be 2.2 x these values
[2] Based on small breed dairy cows because their potential exposure to cyanotoxins is greater.
[3] The short-term RfD is shown for anatoxin-a.

Table 6: Cyanotoxin RfDs and water action levels[1] for beef cattle

	Microcystin	Anatoxin-a	Cylindrospermopsin
Water consumption L/kg-d	0.07	0.07	0.07
Acute RfD mg/kg-d[3]	0.04	0.03	0.04
Acute action level µg/L	**500[2]**	**400**	**600[2]**
Subchronic RfD mg/kg-d	0.0006	0.03	0.003
Subchronic action level µg/L	**8**	**400**	**40**

[1] Calculated as: Action level (mg/L) = RfD (mg/kg-day) / Intake of contaminated water (L/kg-day)
Based on cattle fed dry diet; Action levels for cattle on pasture would be 2.2 x these values
[2] Action levels for microcystin and cylindrospermopsin differ due to rounding in acute RfDs.
[3] The short-term RfD is shown for anatoxin-a.

Foraging on cyanobacterial crusts or mats

Cattle have been known to eat cyanobacterial crusts or mats on the edge of natural or impounded water bodies. This scenario is especially risky considering the high concentrations of cyanotoxins found in cells of cyanobacteria. The extent of this exposure was estimated by utilizing some basic observations in livestock, the details of which are in Appendix VI. Calculated threshold concentrations in dried scum or mats for the intake rate of 1.2 kg/day in cattle are presented in Tables 7 and 8.

Table 7: Acute and subchronic action levels for dairy cow[1] exposure to cyanotoxins in crusts & mats

	Microcystin	Anatoxin-a	Cylindrospermopsin
Algal consumption kg/kg-d	0.0026	0.0026	0.0026
Acute RfD mg/kg-d[2]	0.04	0.03	0.04
Acute action level mg/kg[3]	**15**	**10**	**15**
Subchronic RfD mg/kg-d	0.0006	0.03	0.003
Subchronic action level mg/kg[3]	**0.2**	**10**	**1**

[1] Based on small breed dairy cows, 454 kg, because their potential exposure to cyanotoxins is greater.
[2] The short-term RfD is shown for anatoxin-a.
[3] Based on dry sample weight

Table 8: Acute and subchronic action levels for beef cows' exposure to cyanotoxins in crusts & mats

	Microcystin	Anatoxin-a	Cylindrospermopsin
Algal consumption kg/kg/d	0.0019	0.0019	0.0019
Acute RfD mg/kg/d[1]	0.04	0.03	0.04
Acute action level mg/kg[2]	**20**	**15**	**20**
Subchronic RfD mg/kg/d	0.0006	0.03	0.003
Subchronic action level mg/kg[2]	**0.3**	**15**	**2**

[1] The short-term RfD is shown for anatoxin-a.
[2] Based on dry sample weight

Dogs

Exposures from drinking and grooming

Dogs may be exposed to cyanotoxins by drinking from contaminated water bodies and by licking their coats after swimming in contaminated water. Exercising dogs are estimated to drink up to 0.01 L/kg-hr (see appendix VI). We estimate that 1.5 L of bloom waters may cling to the coat of a 20 kg dog and be ingested during grooming. This is equivalent to 0.074 L/kg (see appendix VI). Thus, the total amount of ingested during drinking (for 1-hr of exercise) and grooming is 0.084 L/kg. Results are in Table 9.

Table 9: RfDs & acute and subchronic action levels for canine exposure to cyanotoxins in drinking water

	Microcystin	Anatoxin-a	Cylindrospermopsin
Water consumption L/kg-d	0.084	0.084	0.084
Acute RfD mg/kg/d[1]	0.04	0.03	0.04
Acute action level µg/L	500	400	500
Subchronic RfD mg/kg/d	0.0006	0.03	0.003
Subchronic action level µg/L	7	400	40

1 The short-term RfD is shown for anatoxin-a.

Foraging on cyanobacterial crusts or mats

Dogs eat large meals and can consume a day's energy requirement in just a few minutes. Active pets require approximately 130 kcal (kg bw)$^{-0.75}$ per day while hunting dogs require approximately 240 kcal (kg bw)$^{-0.75}$ [122]. For dogs playing in and near water bodies the average of these values, 185 kcal (kg bw)$^{-0.75}$ per day, was used. For a 20 kg dog, the energy requirement would be approximately 1750 kcal which would be approximately 0.5 kg of dry dog food [122]. Therefore the potential ingestion of crust material was assumed to be 0.5 kg (0.025 kg/kg-day, Table 10).

Table 10: RfDs & action levels for canine exposure to cyanotoxins in crusts & mats

	Microcystin	Anatoxin-a	Cylindrospermopsin
BGA consumption kg/kg-d	0.025	0.025	0.025
Acute RfD mg/kg-d[1]	0.04	0.03	0.04
Acute action level mg/kg[2]	**1.5**	**1**	**1.5**
Subchronic RfD mg/kg-d	0.0006	0.03	0.003
Subchronic action level mg/kg[2]	**0.02**	**1**	**0.1**

1 The short-term RfD is shown for anatoxin-a.
2 Based on dry sample weight

Summary

Table 11: Human water action levels for various scenarios (µg/L)

	Microcystins	Anatoxin-a	Cylindrospermopsin
Recreational uses	0.7	50	4

Table 12: Sport fish and shellfish action levels for consumption (ng/g)

	Microcystins	Anatoxin-a	Cylindrospermopsin
Sport fish tissue level	13	1100	66

Table 13: Domestic animal water action levels for various scenarios (µg/L)

	Microcystins	Anatoxin-a	Cylindrospermopsin
Acute action level dairy	200	100	200
Subchronic action level, dairy	3	100	10
Acute action level, beef	500	400	600
Subchronic action level, beef	8	400	40
Acute action level, dog	500	400	500
Subchronic action level, dog	7	400	40

Table 14: Domestic animal action levels for cyanotoxin concentrations in crusts and mats (mg cyanotoxin /kg cells dw)

	Microcystins	Anatoxin-a	Cylindrospermopsin
Acute action level dairy	15	10	15
Subchronic action level, dairy	0.2	10	1
Acute action level, beef	20	15	20
Subchronic action level, beef	0.3	15	2
Acute action level, dog	1.5	1	1.5
Subchronic action level, dog	0.02	1	0.1

Appendix I: Determination Swimmer Exposure

This scenario is designed to ensure that people swimming are not exposed to concentrations of cyanotoxins that could cause adverse health effects. Cyanotoxins in the water could theoretically enter the swimmers bloodstream by three routes.

1. Ingestion: Swimmers, especially children, accidentally swallow the water in which they are swimming.
2. Dermal uptake: Some chemicals are absorbed through the skin of swimmers.
3. Inhalation: Volatile chemicals or those in aerosols may be present in the air above the water. The swimmer may inhale these vapors or aerosols while swimming.

Dose from Water Ingestion

Swimmers may inadvertently swallow (ingest) water while swimming. Cyanotoxins in the swallowed water can be absorbed into the blood from the stomach and intestines. The amount of a toxin ingested is proportional to the amount of water that is swallowed, the concentration of chemical in the water, the absorbed fraction, and the time spent swimming, and inversely proportional to the body weight. The absorbed dose is calculated using the following equation:

$$D_{ingest} = \frac{C_w \times ET \times IR \times Abs}{BW} \qquad \text{eq. 1}$$

where:

D_{ingest} = Dose from ingesting water while swimming (mg/kg/event),
ET = Exposure time (hrs/event),
IR = Ingestion rate (L/hr),
C_w = Chemical concentration in water (mg/L),
Abs = Fraction absorbed (assumed to be 100 percent),
BW = Body weight of exposed individual (kg).

Dose from Skin Penetration

Some chemicals can penetrate the skin to reach the blood. The following equation shown below is how the absorbed dose is calculated for those chemicals:

$$D_{dermal} = \frac{C_w \times ET \times SA \times K_p \times R_1 \times R_2}{BW} \qquad \text{eq. 2}$$

where:

D_{dermal} = Dose from dermal penetration while swimming (mg/kg-event)
ET = Exposure time (hrs/event),
SA = Surface area of exposed skin (m^2),

C_w = Concentration in water (mg/L),
K_p = Chemical-specific permeability coefficient (cm/hour),
BW = Body weight of exposed individual (kg).
R_1 = Conversion factor for square meters to square centimeters (10,000 cm^2/m^2)
R_2 = Conversion factor for cubic centimeter to liters (0.001 L/cm^3)

As in all the other equations, the intake dose is proportional to the time swimming (ET), the concentration of chemical in the water (C_w), the surface area of the person (SA), and inversely proportional to the body weight. The absorbed dose is proportional to the dermal permeability coefficient (K_p), a physiochemical property of the chemical indicating its ability to penetrate skin.

Dose from Inhaled Vapors

Volatile chemicals may vaporize from the water into the air above the water. A swimmer would inhale these chemicals while swimming. The following equation shows how the intake dose was calculated.

$$D_{inhaled} = \frac{C_a \times ET \times IR}{BW} \qquad \text{eq.3}$$

where:

$D_{inhaled}$ = Dose from inhaling vapors in air while swimming (mg/kg-event)
C_a = Ambient vapor or aerosol concentration in air (mg/m^3),
ET = Exposure time (hours/event),
IR = Inhalation rate (m^3/hour),
BW = Body weight of exposed individual (kg)

$$C_a = C_w \times H' \times R_3$$

where:

C_w = Concentration in water (mg/L), and
H' = Chemical specific Henry's Law Constant (µg/m^3 air per mg/L water)
R_3 = Conversion factor for micrograms to milligrams (0.001 mg/µg)

The intake dose is proportional to the time spent swimming (ET), the inhalation rate (IR) and the concentration in air (C_a). Air concentrations are predicted using the Henry's Law constant that is a property of the chemical.

Chemical-Specific Considerations

Three standard routes of exposure are described above. However, if the chemical does not penetrate the skin or vaporize from the water into the air, then it does not pose a threat by the dermal or inhalation routes, respectively. Dermal penetration and volatility are related to basic chemical properties. The following table shows the routes of exposure that are assumed to be complete for the four microcystins, cylindrospermopsin and anatoxin-a.

	Microcystins	Cylindrospermopsin	Anatoxin-a
Ingestion	yes	Yes	yes
Dermal	no[1]	no[1]	yes
Inhalation	no[1]	no[1]	yes

[1] Based on their chemical properties, microcystins and cylindrospermopsin are not likely to penetrate the skin or vaporize from water.

Two age groups of children as well as both male and female adults were evaluated to determine which group would receive the highest dose of each of the three chemicals. There are three routes of exposure by which a chemical can travel from swimming water into a swimmers body: ingestion, inhalation and dermal contact. The equations establishing the relationship between the water concentration and the dose a person receives require human-specific parameters as well as chemical-specific parameters. The following two tables show the values that were used in the exposure equations.

Human Parameters			**Different Swimmer Groups**				
			Child 7 to 10	**Child 11 to 14**	**Adult male**	**Adult female**	**Adult both sexes**
Name	**Symbol**	**units**					
Ingestion Rate[1]	IR_{ingest}	l/hr	0.05	0.05	0.025	0.025	0.025
Inhalation Rate[2]	IR_{inhale}	m^3/hr	1	1	1	1	1
Exposure Time	ET	hr	5[3]	3[4]	5[5]	5[5]	5[5]
Body Weight	BW	kg	30.25[6]	48.26[7]	78.17[8]	65.47[8]	71.87[9]
Body Surface Area	SA	m^2	1.041[10]	1.422[11]	1.943[12]	1.693[12]	1.842[9]

[1] ACC, 2002 - based on EPA pilot study
[2] [123] Table 5-23: short-term exposures, light activity.
[3] [123] Table 15-119: 90th percentile value for time spent in an outdoor pool for age 5-11 yrs.
[4] [123] Table 15-119: 90th percentile value for time spent in an outdoor pool for age 12-17 yrs.
[5] [123] Table 15-119: 90th percentile for time spent in an outdoor pool for males or females aged 18-75.
[6] [123] Table 7-3: Average of 7, 8, 9, 10 year old girls & boys.
[7] [123] Table 7-3: Average of 11,12,13,14 year old girls & boys.
[8] [123] Table 7-2: Average of for males or females aged 18-75.
[9] Mean of adult males and females
[10] [123] Tables 6-6 & 6-7: Average of male & female medians for ages 7<8, 8<9, 9<10, & 10<11
[11] [123] Tables 6-6 & 6-7: Average of male & female medians for ages 11<12, 12<13, 13<14, & 14<15
[12] [123] Table 6-4: The 50th percentile value for males or females aged 18-75.

Chemical Parameters			Anatoxin	Cylindrospermopsin	Microsystin
Constant Name	**Symbol**	**Units**			
Skin Permeability[1]	K_p	cm/hr	7.00E-03	NA[3]	NA
Henry's Law[2]	H'	µg/m^3/mg/L	3.58E-05	NA	NA

[1] This is an estimate of the rate at which a chemical will penetrate skin.
[2] Henry's law constants are typically shown as pressure/water concentration. Using the universal gas constant pressure was converted to air concentration (µg/m^3 air)
[3] Not applicable

The skin permeability constant and the Henry's law constant were used to predict the amount of anatoxin that penetrated the skin and concentration in the inhaled air respectively. There is no evidence that either cylindrospermopsin or microcystins leave water for air or penetrate the skin. This is expected because they are large zwitterions. Therefore, the chemical constants were not needed for those two chemicals.

The following table shows the doses (in mg/kg-event) of chemical each group of swimmers would receive if the water contained 1 mg/liter of each of the three chemicals. It also shows that children between the ages of 7 and 10 have the highest exposure to all three chemicals. Therefore, a water concentration that protects this group of swimmers will -protect older children and adults.

Chemical	Exposure route	Child, 7 to 10	Child 11 to 14	Adult male	Adult female	Adult both sexes
Microcystin	Ingestion	**8.26E-06**	3.11E-06	1.60E-06	1.91E-06	1.74E-06
Cylindrospermopsin	Ingestion	**8.26E-06**	3.11E-06	1.60E-06	1.91E-06	1.74E-06
Anatoxin	Ingestion	8.26E-06	3.11E-06	1.60E-06	1.91E-06	1.74E-06
	Inhalation	5.92E-09	2.23E-09	2.29E-09	2.73E-09	2.49E-09
	Dermal	1.20E-05	6.19E-06	8.70E-06	9.05E-06	8.88E-06
	Total	**2.03E-05**	9.30E-06	1.03E-05	1.10E-05	1.06E-05

Exposure Parameters for the Swimmer and the Relationship between Water Concentration and the Dose to Swimmers

Since a child between the ages of 7 and 10 is more exposed than older children or adults, the exposure parameters for 7-to-10-year-old children were used to calculate the values in the table below, which shows the ratios of the concentration of cyanotoxins in water to the swimmers' potential daily dose. These ratios have units of milligrams chemical per kilogram of body weight per milligram of chemical per liter of water. The ingestion ratios for anatoxin-a were computed using equations 1, 2, and 3 for ingestion, dermal absorption, and inhalation, respectively, assuming that a 30.2 kg child swimming for 5 hours a day ingests 50 milliliters of water per hour and breathes one cubic meter of air per hour. For microcystins and cylindrospermopsin only equation 1 was used because these chemicals are not volatile and do not penetrate the skin to any significant degree (see below).

Ratios of Swimming Water Concentration over Swimmer Dose (mg/L)/(mg/kg)

Chemical	Ingestion	Inhalation	Dermal	Total[a]
Microcystins	1.21×10^{2}	none	none	1.21×10^{2}
Cylindrospermopsin	1.21×10^{2}	none	none	1.21×10^{2}
Anatoxin-a	1.21×10^{2}	1.69×10^{2}	5.61×10^{2}	1.05×10^{2}

[a] $$\text{Total} = \frac{1}{\frac{1}{\text{Ingestion}} + \frac{1}{\text{Inhalation}} + \frac{1}{\text{Dermal}}}$$

Health-Based Water Concentrations

The Concentration/Dose Ratios for each of the chemicals were multiplied by the corresponding RfD (in Section III) to estimate an Action Level, a water concentration that would theoretically expose the child swimmer to the dose identified as the maximum dose to which a person may be exposed with little to no risk of harm. The Action Levels are shown as micrograms (µg) per liter. A microgram is 1/1000 (0.001) of a milligram.

Cyanotoxin Action Levels for the Swimming Scenario

Chemical	RfD[a]	Concentration/Dose Ratio[b]	Action Level[c]
Units	mg/kg-d	(mg/l) per (mg/kg-d)	µg/L
Microcystins	6×10^{-6}	1.21×10^{2}	0.7
Cylindrospermopsin	3×10^{-5}	1.21×10^{2}	4
Anatoxin-a	5×10^{-4}	1.05×10^{2}	50

[a] The Reference Dose is the maximum dose to which a person should be exposed. The derivation is shown in the body of this document.
[b] These ratios are taken from the table above
[c] The Action Level is the product of the RfD and the Concentration Dose Ratio

Volatility and Skin Permeability of Cyanotoxins

Microcystin molecules are very large relative to volatile chemicals and the carboxylic acids are negatively charged at the pH of normal surface waters. Due to their size and charge, there is little likelihood of these molecules vaporizing into the air from water. Therefore, the inhalation pathway was eliminated.

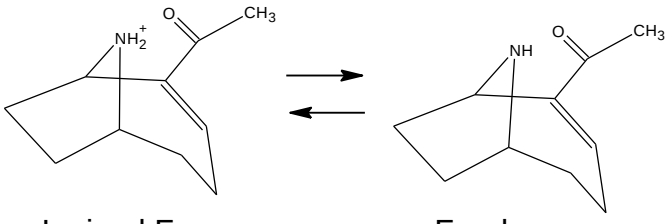
No studies of dermal absorption of microcystins could be found. However, the antibiotics cyclosporin and bacitracin are large cyclic peptides with a chemical structure

similar to microcystins. There have been several attempts to formulate these antibiotics with carriers to help them penetrate skin and all have failed [124-128]. Some authors have suggested that high molecular weight chemicals like microcystins cannot penetrate the skin [129]. Furthermore, chemicals that dissolve easily in water or are charged tend not to penetrate the skin. Like microcystins, these antibiotics are relatively water soluble. Therefore, the dermal exposure route was not assessed for microcystins.

At a molecular weight of 415, cylindrospermopsin is a relatively large molecule. It is also a zwitterion given both the negative charge (associated with the sulfoxy group) and the positive charge (associated with the resonance stabilized guanidine carbon). Large molecules, especially zwitterions, do not volatilize into the air out of water. No information on dermal absorption could be obtained. But due to its large size and charged nature, like microcystins, it was assumed not to penetrate the dermis. Therefore, the inhalation and dermal pathway were eliminated.

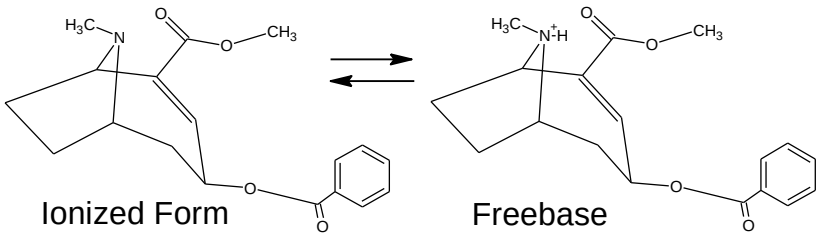
Anatoxin-a differs from the microcystins and cylindrospermopsin. It is not as large a molecule as the other cyanotoxins. Therefore, it was assumed that it could both volatilize and be absorbed through the skin. The amount of anatoxin-a above water is described by the Henry's law constant (H') for anatoxin-a. The amount of anatoxin that penetrates the skin from the water is described by the skin permeability (K_p) of anatoxin-a. Finding H' and K_p for anatoxin-a is complicated by two factors. First, these parameters are actually measured for very few chemicals and therefore are usually estimated using equations or surrogate chemicals. Second, anatoxin-a has an ionizable nitrogen and can therefore exist in a charged and uncharged form.

The Henry's law constant can be estimated as the vapor pressure divided by the water solubility of a chemical [130]. Unfortunately, neither a solubility nor a vapor pressure could be found for anatoxin-a. However, cocaine has a very similar structure to anatoxin-a, although it is a somewhat larger molecule (see comparison below). Both anatoxin-a and cocaine exist in free base and ionized forms.



Ionized Form Freebase

Structure of anatoxin-a



Structure of cocaine

Both a solubility and a vapor pressure have been reported for both the free base and the ionized form of cocaine.

	Cocaine Ionized	Cocaine Free Base
Vapor Pressure[1] (torr)	1.40E-08	2.96E-07
Solubility[2] (gm/liter)	2000	1.7
Henry's Law[3] torr/(gm/liter water)	7.00E-12	1.74E-07
Henry's Law[4] (µg/m^3 air)/(mg/liter water)	1.44E-09	3.58E-02

[1] Dindal et al. 2000
[2] InChem 2007
[3] Ratio of vapor pressure/solubility (Lyman et al. 1990)
[4] Torr converted µg/m^3 using Universal Gas Constant assuming 20 C

As expected, the ionized form of cocaine is much less volatile and more water soluble than the free base. At equal concentrations in water there would be more than 20,000 times as much free base as ionized cocaine in the air. The ratio of ionized to free base in water depends on pH. For this exposure estimate the assumption made was that all of the anatoxin-a is in the form of the free base with a Henry's Law Constant equal to that of the cocaine free base. This greatly overestimates the concentration in air, but is health-protective.

A skin permeability constant (K_p) could not be found for anatoxin-a. Nor could a K_p be found for a structurally similar chemical. Therefore, the following equation was used to estimate K_p [131]

$$K_p = 0.0019 \times K_{ow}^{0.71} \times 10^{(-0.0061*MW)}$$

This equation requires an octanol-water partition coefficient (K_{ow}) and a molecular weight (MW). The molecular weight of anatoxin-a is 165.2 grams/mole. No K_{ow} could be found for anatoxin-a, but Berfield et al.[132] reported a K_{ow} of 303 for the free base of cocaine. A K_p of 0.01079 cm/hour was calculated a using the above equation.

Appendix II: Computation of Action Levels in Sport Fish and Shellfish

Exposure Scenarios Considered

In California, the general fishing population is estimated to consume about 30.5 grams of sport fish and shellfish per day (weighted average of the Santa Monica Bay Seafood Consumption Study; [119], [120]. This consumption rate is equivalent to an uncooked 7.5-ounce fillet each week, which is slightly smaller than the 8-ounce meal size typically used in risk assessments [121]. In order to simplify the action level calculated here, the consumption rate was adjusted to 32 g/day (8 oz/week; uncooked) to reflect a standard meal size.

The exposure scenario for children is also one meal per week, however the meal sizes are assumed to be smaller. USEPA-recommended multipliers were used to convert adult consumption levels to consumption by younger individuals. These multipliers simply reduce meal size in proportion to body weight. The resulting consumption rates for children are similar to typical values observed in consumption surveys [120].

Table 1. Consumption rates used in exposure assessment

Human Parameters		Different Consumer Groups			
Name	**Units**	**Child 0 – 6**	**Child 6 - 9**	**Child 9 – 12**	**Adult**
Consumption[1] Rate	g/day	7	12	17	32
Multiplier[2]		0.21	0.36	0.51	1
Body Wt[3]	Kg	14.5	25	36	70
Consumption Rate	g/kg/d	0.48	0.48	0.47	0.46

[1] Child consumption rate is the product of adult consumption rate and the multiplier.
[2] USEPA-recommended multipliers reduce meal size in proportion to body weight.
[3] Child body weights taken from [121].

Table 2. Adjusted meal size for children[1]

Age (yr)	Meal Size (oz, uncooked)
0 – 6	**2**
6 – 9	**3**
9 – 12	**4**

[1] Based on the calculations shown in Table 1.

Dose from Sport Fish and Shellfish Consumption

Chemicals in the ingested food can be absorbed into the blood from the stomach and intestines. For our purpose, we assume that all ingested cyanotoxin is absorbed from the intestines. The absorbed dose is calculated using the following equation:

$$D_{consume} = \frac{C_F \times CR \times Abs}{BW} \qquad \text{eq. 1}$$

where:

$D_{consume}$ = Dose from consumption of sport fish and shellfish (mg/kg/day),
CR = Consumption rate (g/day),
Abs = Fraction absorbed (assumed to be 100 percent),
C_F = Chemical concentration in edible fish and shellfish tissues (mg/g),
BW = Body weight of exposed individual (kg).

To determine the action level for each cyanotoxin in fish and shellfish, the equation above was rearranged to solve for the concentration in fish and shellfish (C_F) where the dose from consumption ($D_{consume}$) is equal to the RfD, the consumption rate (CR) is 32 g/day (which is equal to one 8 ounce uncooked fillet each week) and the body weight (BW) is 70 kg (typical of an adult).

$$C_F = \frac{RfD \text{ x } BW}{CR \text{ x } Abs} \qquad \text{eq. 2}$$

where:

C_F = Chemical concentration in edible fish and shellfish tissues (mg/g),
RfD = (mg/kg-day),
Abs = Fraction absorbed (assumed to be 100 percent),
CR = Consumption rate (32 g/day),
BW = Body weight of exposed individual (70 kg).

Table 3. Action level based on the consumption of one fish meal per week

Chemical	RfD	C_F[1]	Action Level[2,3]
Units	mg/kg-d	mg/g tissue	ng/g tissue
Microcystins	6×10^{-6}	1.3×10^{-5}	13
Cylindrospermopsin	3×10^{-5}	6.6×10^{-5}	66
Anatoxin-a	5×10^{-4}	1.1×10^{-5}	1100

[1] Chemical concentration in edible fish and shellfish tissues, calculated as shown above in eq. 2.
[2] Converted from C_F (mg/g) by multiplying by 1×10^6 ng/mg
[3] Based on consumption rate of 32 g/day (one 8 oz. uncooked fillet per week) and body weight of 70kg.

The action levels are based on the adult exposure level because this is, by far, the most comprehensive data set. However, action levels calculated for child consumption scenarios (Tables 1 & 2) are within 95% of the adult action levels. Therefore, the action levels presented above are protective of the adult and child consumption scenarios considered here (Tables 1 & 2). For higher consumption rates, these action levels should be divided by the average number of meals ingested per week. Adult meals are assumed to be an 8-ounce fillet (uncooked; [121]). Children's meals are assumed to consist of 2 to 4 ounces (uncooked; see Table 2).

Appendix III: Computation of Microcystin Concentrations in Lyophilized *M. Aeruginosa* Cells used by Jackson et al., 1984.

For the purpose of this risk assessment in domestic animals, the microcystin dosages used by Jackson et al. [86] were estimated using data from this and other studies. Jackson et al. [86] reported the mouse i.p. LD_{100} of the lyophilized *M. aeruginosa* as 19 mg/kg. In a related study, Ellman et al. [133] found that *M. aeruginosa* bloom material from a similar location, during a previous year, showed a mouse i.p. LD_{100} of 15 – 30 mg/kg, which coincides with the findings by Jackson et al. [86]. Ellman et al. [133] also showed that the toxin purified from the *M. aeruginosa* bloom material had an i.p. LD_{50} and LD_{100} of 56 and 70 µg toxin/kg in mice, respectively. A typical i.p. LD_{50} value for MC-LR in mice is 55 µg/kg [2], which is very close to that of the toxin purified from the bloom material in Ellman et al. [133]. Additionally, the i.p. LD_{100} of the purified toxin was only a 25% increase over the i.p. LD_{50}, which agrees with the findings of Lovell et al, [94] for MC-LR. The above indicates that the *M. aeruginosa* used by Jackson et al. [86] had similar toxicity to the *M. aeruginosa* used by Ellman et al. [133], which was likely due to MC based on the i.p. LD_{50} and LD_{100} of the purified toxin. Therefore, we estimated the microcystin dose levels used by Jackson et al. [86] by equating the measured mouse i.p. LD_{100} of 19 mg lyophilized *M. aeruginosa* /kg bw to an estimated mouse i.p. LD_{100} of 69 µg MC/kg bw, which is a 25 percent increase over the typical murine i.p. LD_{50} for MC-LR. The oral lethal NOAEL of 1.01 g lyophilized *M. aeruginosa*/kg bw in sheep is converted to 3.7 mg MC/kg bw as shown below.

Set mouse i.p. LD_{100} for MC-LR equal to murine i.p. LD_{100} for lyophilized *M. aeruginosa* and solve for the amount of microcystin in 1 mg lyophilized *M. aeruginosa*:

$$MC = \frac{PMC}{LMC} \times NOAEL = \frac{0.069 \text{ mg/kg bw}}{19 \text{ mg/kg bw}} \times 1010 \text{ mg/kg bw} = 3.7 \text{ mg/kg bw}$$

where,

MC = Sheep oral lethal NOAEL of purified MC-LR, in mg/kg bw
PMC = Mouse i.p. LD_{100} for purified MC-LR, in mg/kg bw
LMC = Mouse i.p. LD_{100} for lyophilized *M. aeruginosa*, in mg/kg bw
NOAEL = Sheep oral lethal NOAEL of lyophilized *M. aeruginosa*, in mg/kg bw

Appendix IV: Computation of Water Intake by Cattle and Related Action Levels

In general, the total daily water intake (TWI) in cattle is the sum of free water intake (FWI, from drinking) and water ingested from food [134]. Exposure in dairy cows is considered separately due to the dramatic increase in daily water requirements during lactation.

A. Dairy Cattle

Both large- (e.g., Holstein) and small- (e.g., Jersey) breed dairy cows in early- and mid- lactation are considered in this assessment. The FWI of dairy cows at mild to high ambient temperatures was estimated using recommended prediction formulas and dietary parameters from the National Research Council (NRC) [134]. Specifically, the FWI was predicted using the following empirical equation by Murphy et al. [135] as reported by [134]):

$$FWI = 15.99 + 1.58 \times DMI + 0.90 \times FCM + 0.05 \times Na_{intake} + 1.20 \times Temp_{min.}$$

where,

FWI = Free water intake, i.e., drinking (kg/d),

DMI = Dry matter intake (kg/d),

FCM = 4% Fat corrected milk production (kg/d),

Na_{intake} = Sodium ingested through diet (g/day), and

$Temp_{min}$ = Minimum ambient temperature (°C).

Dry matter intake (DMI) and fat corrected milk production (FCM) values for dairy cattle at neutral temperatures (5 - 20 °C) were taken from the nutrient requirement tables in NRC [134]. For higher temperatures, the DMI and FCM values were adjusted as follows.

$DMI_{high\ temp} = DMI \times (1 - (C - 20) \times 0.005922)$ ([136] as reported in [134]).

and

$$FCM_{high\ temp} = \left(\frac{DMI_{high\ temp}}{1 - e^{(-0.192 \times (WOL + 3.67))}}\right) - \left(\frac{0.0968 \times BW^{0.75}}{0.372}\right)$$, rewritten from DMI equation [134],

where,

FCM = 4% Fat corrected milk production (kg/d),

DMI = Dry matter intake (kg/d),

WOL = Week of lactation, and

BW = Body weight (kg).

Sodium intake (Na_{intake}) was based on NRC recommendations for lactating cows as follows:

Ambient Temperature (°C)	Na requirement (g /kg bw/day)
5 – 20	0.038
25 – 30	0.039
> 30	0.043

Minimum ambient temperature ($Temp_{min}$) values were chosen to represent warm summer months, when cyanobacteria blooms are most common.

Table 1. Estimates of free water intake (FWI) for large and small breed dairy cows.

Live Weight (kg bw)[1]	Lactation[2]	Ambient Temp °C	DMI (kg/day)[3]	FCM (kg/day)[4]	FWI (L/day)[5, 6]
454	Early	20	9.4	15	69
454	Early	27	9.0	11	73
454	Early	35	8.6	9	81
454	Mid	20	19.5	30	99
454	Mid	27	18.7	27	103
454	Mid	35	17.8	24	109
680	Early	20	15.1	30	92
680	Early	27	14.5	24	94
680	Early	35	13.8	21	100
680	Mid	20	28.1	45	126
680	Mid	27	26.9	41	129
680	Mid	35	25.6	37	133

[1] Large and small breed dairy cows represented by 680 and 454 kg body weight (bw), respectively.
[2] Early- and mid-lactation estimated at 11 and 90 days of lactation, respectively.
[3] Dry matter intake (DMI).
[4] Fat corrected milk production (4%) (FCM).
[5] Free water intake (FWI).
[6] Sodium intake based on NRC recommendations for lactating cows:

Lactation status and ambient temperature appear to be the strongest factors controlling FWI in cattle during warmer periods. An ambient temperature of 27 °C was found to be most representative of dairy farms in California. The average reported maximum temperatures for California counties with dairy operations during 1970 – 2000 was 28.7 °C (north) and 30.7 °C (south) for dairy farms [137]. Mid-lactation was chosen to represent dairy cows since water intake is greatest during that period.

B. Beef Cattle

The TWI values for mature beef cattle (~635 kg) at mild to high ambient temperatures were taken from nutrient requirement tables in NRC [138]. The FWI of cattle receiving dry diets is generally 83 percent of their TWI [134]. Estimated FWI values are shown in Table 2.

Table 2. Estimated free water intake (FWI) by mature beef cattle on a dry diet

Live Weight (kg bw)	Ambient Temperature (°C)	TWI (L/kg-day)[1]	FWI (L/kg-day)[2]
635	21	0.07	0.06
635	27	0.08	0.07
635	32	0.11	0.09

[1] Total Water Intake (TWI) for mature beef cattle (635 kg) at varying ambient temperatures was taken from the nutrient requirement tables in NRC [138].

[2] Free water intake (FWI). Estimated as 83% of TWI [134]. It is assumed that the entire amount of FWI may originate from a contaminated source.

An ambient temperature of 27 °C was chosen to represent typical cattle ranches in California. Average reported maximum temperatures for California counties with beef cattle operations during 1970 – 2000 was 28.6 °C [137].

C. Effect of Diet and Water Access

The drinking rates (FWI) described above pertain to cattle fed dry diets that are typical of NRC recommendations [134, 138]. In such cases, the entire amount of FWI may originate from a contaminated source. A separate scenario is considered for pasture grazing cattle. Higher moisture content in food leads to decreased FWI [134]. The FWI of pasture grazing cattle is estimated as 38% of TWI [134]. To estimate the FWI for a pasture scenario, TWI values for mature beef cattle (~635 kg) at ambient temperature of 27 °C were taken from NRC nutrient requirement tables [138]. Because the TWI values of dairy cows were not available in NRC reference tables, they were estimated using data shown in Table 1 for dairy cows in mid-lactation at ambient temperature of 27 °C. In general, the total daily water intake (TWI) in cattle is the sum of free water intake (FWI, from drinking) and dietary water intake (DWI, from eating) [134]. DWI can be estimated based on the dry matter intake (DMI) and a dietary moisture level of 30%, which represents most dry diets [134]. DWI was estimated for dairy cows as follows.

$$DWI = \text{Total Diet} \times \%\,\text{Moisture}$$

and

$$\text{Total Diet} = \left(\frac{\text{DMI}}{(1 - \%\,\text{Moisture})} \right)$$

then

$$\text{DWI} = \left(\frac{\text{DMI} \times \%\,\text{Moisture}}{(1 - \%\,\text{Moisture})} \right)$$

where,

DWI = Dietary water intake, or water ingested by eating (kg/d),

Total Diet = Sum of dry matter and water in diet (kg), and

DMI = Dry Matter Intake (kg/day)

% Moisture = Percentage of water in diet (%).

TWI is then estimated for dairy cows as follows.

$$\text{TWI} = \text{FWI} + \text{DWI}$$

where,

FWI = Free water intake, or water ingested by drinking (kg/d),

TWI = Total water intake, or water ingested by drinking and eating (kg/d), and

DWI = Dietary water intake, or water ingested by eating (kg/d).

This scenario also assumes that the pasture-fed cattle mainly drink from the natural or impounded water body. The estimated FWI values for pasture-fed cattle are shown in Table 3.

Table 3. Estimates of free water intake (FWI) for pasture-fed beef and dairy cattle

Livestock Category[1]	TWI (L/kg-day)[2]	Fraction of TWI as FWI	FWI (L/kg-day)[3]
Dairy, small breed	0.24	0.38	0.09
Dairy, large breed	0.21	0.38	0.08
Beef, mature	0.08	0.38	0.03

1 Large and small breed dairy cows represented by 680 and 454 kg body weight (bw), respectively. Values represent mid-lactation. Mature beef cattle based on 635 kg bw. All values reflect ambient temperature of 27 °C.

2 Total Water Intake (TWI) for beef cattle was taken from the nutrient requirement tables in NRC [138]. TWI for dairy cattle was estimated as described above.

3 Free water intake (FWI) for pasture-fed cattle. Estimated as 38% of TWI [134].

An additional exposure scenario is needed for cattle that have short-term access to natural or impounded water bodies. Cattle reportedly drink an average of 7 – 14 times a day, with ingestion rates of 4 - 15 L/min [134]. By assuming that drinking frequency is ten

drinks per day and that up to three occurrences take place during short-term access to natural or impounded waters, an estimated 30% of FWI may originate from a contaminated water source. The estimated water intake values for cattle having short-term access to contaminated water are shown in Table 4.

Table 4. Estimated intake rates of contaminated water in cattle with short term access to contaminated water.

Livestock Category[1]	FWI (L/kg-day)[2]	Intake of Contaminated Water (L/kg-day)[3]
Dairy, small breed	0.23	0.07
Dairy, large breed	0.19	0.06
Beef, mature	0.07	0.02

[1] Large and small breed dairy cows represented by 680 and 454 kg body weight (bw), respectively. Mature beef cattle based on 635 kg bw.
[2] Free water intake (FWI) from Tables 1 and 2. Values represent an ambient temperature of 27 °C and, for dairy cows, mid-lactation.
[3] Estimated as 30% of FWI as described above.

D. Summary of Exposure to Cattle through Drinking

Below are the estimated intake rates of natural or impounded waters for dairy and beef cattle that are 1) mainly fed dry diets, 2) mainly pasture-fed and 3) have only short-term access to natural or impounded waters. The FWI intake values shown below correspond to an ambient temperature of 27 °C. Values for dairy cows reflect mid-lactation status.

Livestock Category[1]	Livestock Scenario[2]	Water intake from contaminated source (L/kg-d)
Dairy, small breed	Dry diet	0.23
	Pasture-fed	0.09
	Short-term	0.07
Dairy, large breed	Dry diet	0.19
	Pasture-fed	0.08
	Short-term	0.06
Beef, mature	Dry diet	0.07
	Pasture-fed	0.03
	Short-term	0.02

[1] Large and small breed dairy cows represented by 680 and 454 kg body weight (bw), respectively. Values represent mid-lactation. Mature beef cattle based on 635 kg bw. All values reflect ambient temperature of 27 °C.

[2] Assumes that the cattle mainly drink from the natural or impounded water body. Livestock scenarios are described in the text above.

E. Action Levels

The action levels were calculated to identify the concentration of cyanotoxins in water that pose very low or no risk of acute or subchronic toxicity to dairy cows or cattle. Action levels are calculated as follows:

$$C_{tot} = \frac{RfD \times BW}{IR}$$

where,

C_{tot} = Concentration of cyanotoxin in total water (µg/L),

RfD = Risk reference dose for domestic animals (µg/kg-day),

BW = Body weight (kg), and

IR = Water intake from affected water body (L/day), based on cattle fed dry diet.

Appendix V: Ingestion of Cyanobacterial Crusts by Cattle

The amount of bloom material a cow would choose to eat can not be predicted. Therefore, the extent of this exposure was esimated by utilizing some basic observations in livestock. The average cow is reported to consume up to 20 spontaneous meals each day [139]. The length of each spontaneous meal is approximately 5 minutes and consists of about 600 g. This exposure assessment is based on the assumption that a cow will eat two spontaneous meals a day of bloom material, i.e. 1.2 kg. Therefore, cattle risk action levels for the concentration of cyanotoxin in cyanobacterial cells (C_{cell}) present as crust or mats can be estimated as follows:

$$C_{cell} = \frac{RfD \times BW}{IR_{crust}}$$

where,

C_{cell} = Cyanotoxin concentration in cyanobacteria cells (mg toxin/kg cells dry weight)

RfD_{cattle} = Risk reference dose for cattle (mg/kg/day)

BW = Body weight of cattle (kg)

IR_{crust} = Ingestion rate of cyanobacterial crust or mats (kg dry weight/day)

Appendix VI: Canine Exposure to Cyanotoxins in Water

Dogs may be exposed to cyanotoxins, by drinking from natural or impounded water bodies. To quantify this possible exposure, the amount of water lost by a dog exercising in a warm environment was estimated. Dogs running on a treadmill at 7 – 10 km/hr lost 2 – 7 ml water/kg body weight (bw)-hour [as reported in 122, 140]. In elevated temperatures, dogs lose approximately 6 ml/kg bw-day for every degree C above 30 °C ([141] as reported in [122]). This would result in an additional 48 ml/kg bw-day at 38° C, equivalent to 2 ml/kg bw-hr. When added to the baseline water loss, this would yield a total water loss of 4-9 ml/kg bw-hr. Thus, a 20 kg dog would require approximately 80 - 180 mL of water per hour during exercise at 38 °C. Although actual water intake may differ based on activity and dehydration levels, as well as other factors, an assumption was made that an exercising 20 kg dog's drinking water intake will be 180 mL of water per hour. The potential hourly exposure by drinking was rounded to 0.2 L, or 0.01 L/kg-hr.

As dogs swim or play in contaminated waters, their coats become saturated with algal cells that may later be consumed during grooming. Dogs often shake off much of the water in their coats, but the algal cells can be filtered by the hair and left behind. To estimate the amount of cyanotoxin that may be contained in the coat of a dog, an assumption is made that the amount of toxin remaining on a dog's coat is equal to that contained within a 2 mm layer of water covering the body surface of the dog. The average body surface area of a 20 kg dog is 0.74 m^2 [142]. Thus, an upper estimate of the volume of water that is representative of the amount of cyanotoxin retained within the coat is calculated to be 0.74 m^2 x 0.002 m = 0.00148 m^3 or 1.48 L. On a body weight basis, the exposure is calculated as 1.48 L ÷ 20 kg = 0.074 L/kg. Based on these estimates, the assumed daily exposure to cyanotoxin due to drinking and grooming is equal to the amount of toxin contained in 0.084 L water/kg bw. This daily exposure represents the total from one coat cleaning plus the replacement water for one hour of exercise.

Appendix VII: Ecotoxicology of Microcystins, Anatoxin-a and Cylindrospermopsin

Introduction

The ecotoxicology of cyanobacterial blooms is a complex and evolving subject. The fact that toxic impacts are merely a subset of the greater ecological disturbances caused by these blooms further complicates this subject. Hundreds of studies on various aspects of cyanobacterial blooms and their toxins (cyanotoxins) are published in the scientific literature. The purpose of this report is to identify key factors in the current understanding of commonly observed cyanotoxins that may assist government scientists and regulators in the protection of aquatic animals. Three of the most commonly observed cyanotoxins are addressed: microcystins, anatoxin-a, and cylindrospermopsin.

Cyanobacterial blooms occur worldwide in fresh and salt waters [2, 143]. Such blooms can change community structure and food web dynamics through a myriad of potential pathways including, for example, changes to essential habitat parameters (shading, decreased dissolved oxygen and pH), decreased flow of carbon into food webs and toxic effects on aquatic life [reviewed by 144, 145].

Blooms are often recognized by the presence of thick blue-green surface scums. However, some species of cyanobacteria, such as *Cylindrospermopsis* spp., proliferate into low-biomass, but toxic, blooms. Benthic proliferations typically appear as dense mats on the sediment and submerged rocks [19, 40]. Blooms can last from months to year-round depending on local conditions [reviewed in 2].

The occurrences of cyanobacterial blooms appear to be increasing [reviewed by 146]. The geospatial range of several species has also increased in recent decades [147]. Possible explanations are increased nutrient loading due to human activities [148], increasing global temperatures [149] and increased monitoring and reporting [143].

Paerl et al. [144] provide an in-depth review of the causes of cyanobacterial blooms. In general, factors supporting bloom formation include high turbidity, warmer temperatures, increased nutrients and water residence time (stagnation). However, toxic blooms also occur in cold and oligotrophic (nutrient poor) waters [e.g., 40]. A large body of literature exists on the biology and occurrences of cyanobacterial blooms. Excellent reviews of this subject are provided by WHO [2], Paerl et al. [144] and Sinclair et al. [143].

Toxic impacts on aquatic organisms

Toxic blooms of cyanobacteria pose a significant threat to organisms associated with aquatic ecosystems. Cyanotoxins in bloom material commonly reach highly toxic concentrations [reviewed in 2]. Animals are exposed to cyanotoxins by directly ingesting cyanobacterial cells or consuming other organisms that have recently ingested cyanobacteria. Cyanotoxins that have been released from cyanobacteria into the surrounding water are also taken up by aquatic organisms, but to a lesser extent. Field and laboratory studies show that aquatic organisms can accumulate high levels of cyanotoxins in their tissues. The effects of these toxins have mainly been demonstrated through laboratory experiments, although some field experiments are described.

Several review papers address cyanotoxin aquatic toxicology. Wiegand and Pflugmacher [150] provide a succinct biochemical review of cyanotoxins with a focus on aquatic animals. Duy [151] provides a comprehensive review of the toxicology of cyanotoxins. Carmichael [152-154] has published several important reviews on this subject. A detailed review of toxic effects of microcystins in fish (and some amphibians) is provided by Malbrouck and Kestemont [155]. Ibelings and Havens [156] perform a qualitative meta-analysis of the exposures and effects of cyanotoxins in aquatic animals. Landsberg [30] published an extensive review of observed impacts on fish and wildlife (mostly lethal) coinciding with cyanobacterial blooms.

Some topics pertaining to the ecotoxicology of cyanobacteria are beyond the scope of this report. For example, many cyanobacterial species are capable of allelopathy,

whereby toxins are released that damage other species of cyanobacteria [157], algae [158-160] and plants [161, 162]. Interaction between cyanobacteria and zooplankton communities is another large area of study and is only partially addressed here. More information on the impacts of cyanobacterial blooms on aquatic organisms and ecosystems can be found in Carmichael [163], WHO [2], Paerl et al. [144] and Havens [145].

Terminology

The terminology used in cyanotoxin literature can be confusing. In experiments, animals are exposed to cyanotoxins as whole cyanobacteria, extracts of cyanobacteria or pure toxins. Animals are exposed to cyanobacteria that is suspended in water, filtered from water (cells) or dried. Extracts and pure toxins are typically dissolved in water. Concentrations of cyanotoxin are reported as toxin in cells and water (total), cells only (intracellular) or water only (dissolved or extracellular). These terms are described below.

Terminology

Common preparations of cyanotoxins used in experiments	
Bloom or scum material	Fresh or freeze-dried cyanobacteria collected from a naturally occurring bloom.
Cultures	Cyanobacteria grown in an artificial setting. Some cultures contain a single strain of a cyanobacterial species.
Crude extracts	Broken cells (e.g., by sonication) that have been centrifuged to remove debris.
Cell-free extracts	Crude extracts that have been filtered to remove remaining cell fragments.
Extracts	Broken cells (e.g., by sonication) are extracted with solvent and filtered from cell fragments. Further clarification using solid phase extraction may also take place.
Purified toxin	A single toxin isolated from extracts, typically using high performance liquid chromatography.
Pure toxin	Commercial grade toxins.

Terminology (continued)

Common forms of cyanotoxins used in experiments		
Form	**Description**	**Unit of Measurement**
Dissolved	Toxin or extract is dissolved in water.	Microgram toxin per liter of water (µg/L).
Natural or cultured cyanobacteria (fresh)	Fresh cells of cyanobacteria.	Microgram toxin in cells per liter of filtered cells (µg/L).
Natural or cultured cyanobacteria (dried)	Freeze-dried cells of cyanobacteria.	Microgram toxin in cells per gram of dried cells (µg/g).
Suspensions of cyanobacteria (fresh or dried)	Cells of cyanobacteria suspended in water.	Microgram toxin (in cells and in water) per liter of the water and cell mixture (µg/L).

Terminology (continued)

Measurements of cyanotoxins		
Form	**Description**	**Unit of Measurement**
Dissolved	Toxin concentration in water: • extracellular toxin concentrations in natural waters (i.e., toxin released from cyanobacterial cells) • pure toxin dissolved in water • extracts dissolved in water	Microgram toxin per liter of water (µg/L).
Intracellular	Toxin concentration in cyanobacterial cells.	Microgram toxin in cells per liter of wet cells (µg/L), or per gram of dry cells (µg/g).
Total	Toxin concentration in cyanobacterial cells and in water. Used in measurements of experimental algal suspensions or of natural bloom waters.	Microgram toxin (in cells and in water) per liter of the water and cell mixture (µg/L).
Tissue	Toxin concentration in animal tissue.	Microgram toxin per gram of animal tissue (µg/g). The concentration is based on tissue wet weight (ww) or dry weight (dw).

Terminology (continued)

Measurements of microcystin congeners and metabolites	
Congener or metabolite	Description
MC-LR equivalents	The analysis was calibrated using a MC-LR standard.
Specific MC congeners	The analysis was calibrated using specified congener standards.
Free microcystins	The typical analyte in microcystin analyses. Microcystin that is not bound to a bioactive molecule such as protein phosphatase or glutathione. Measured in cyanobacteria, water and biological tissues. (Note, the terms dissolved or extracellular, and not free-MC, are used to distinguished microcystins that are 'free in the water' as opposed to contained inside cyanobacterial cells).
Covalently bound microcystins	Microcystin that is bound covalently to protein phosphatase. Measured only in biological tissues. Requires a special analysis that is not typically used.

Microcystins

Introduction

Aquatic organisms primarily take up microcystins through ingestion of food [156]. In general, these animals show greater impacts when exposed to crude extracts of cyanobacteria compared to purified microcystin [164]. This is due to the actions of other cyanobacterial compounds in crude extracts. Nevertheless, purified microcystin does exert significant toxicity in aquatic animals.

All aqueous microcystin concentrations reported here include the toxin within the cyanobacterial cells in addition to any dissolved toxin in the water (or ***total*** concentration) unless concentrations are specifically identified as ***dissolved*** (toxin dissolved in water, but not within cells).

Toxic Mechanism

The mechanism of toxic action by microcystins has been well described elsewhere [151]. In brief, the 3-amino-9-methoxy-2-6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) moiety of microcystins enters the hydrophobic cleft of protein phosphatases 1 and 2A (PP1 and 2A), inhibiting the activity of these critical cellular enzymes. Inhibition of PP1 and 2A interferes with normal cellular function and leads to cell death. Microcystin can also lead to oxidative damage in cells [165, 166]. The liver is the main target organ in most animals.

Congeners

There are over 70 congeners of microcystin [167]. The general structure and nomenclature of microcystins are described in the main report (Section II). Unless a specific congener is listed, all microcystin (MC) concentrations are reported here as microcystin-LR equivalents (i.e., MC-LR was the only standard used in analysis). Most research has focused on microcystin-LR, which shows the greatest toxic potency when injected into mice [reviewed in 2]. However, aquatic organisms also accumulate other microcystin congeners [168-170]. The relative toxicity of microcystin congeners has been studied based on structure-function relationships [171], relative hydrophobicity [172] and zooplankton bioassays [173]. Recently, the combined use of protein phosphatase inhibition assays, mouse bioassays and molecular structure analysis have provided additional insight [174, 175]. Although microcystin-LR is regarded as the most toxic congener (based on mouse injection bioassays), it is important to realize that the relative toxicities of microcystin congeners are only beginning to be understood. For example, one type of microcystin-RR congener ([D-Asp(3),(E)-Dhb(7)]MC-RR) produced greater toxicity in zooplankton compared to microcystin-LR, -RR and -YR, which produced similar toxicities [173]. More work is needed in order to understand the relative toxicity of microcystin congeners to aquatic organisms.

Different microcystin congeners may also move through the food web differently. Not all microcystin congeners form covalent bonds with PP-1 and -2A. Congeners containing methyldehydroalanine (Mdha) bind PP-1 and -2A covalently, while those containing dehydrobutyirine (Dhb) do not [156, 176, 177]. Microcystin congeners that do not form covalent bonds are suspected to transfer through the food web more efficiently than covalently binding congeners, but this has not been tested [156, 178].

Detoxification

Glutathione (GSH) can conjugate microcystins with the aid of glutathione-s-transferase [179, 180], which leads to reduced toxicity [179, 181] and excretion via bile [182]. Many aquatic organisms have been shown to utilize GSH in microcystin detoxification including brine shrimp [183], water fleas [180], mussels [168, 180, 184], crabs [185] and fish [180, 186]. Aquatic species differ in their capacity to detoxify microcystins through the GSH pathway [187].

Blooms

Cyanobacterial bloom material has been shown to contain microcystin concentrations up to 12,800 µg/g dry weight (dw) [188] and 25,000 µg/L wet volume [reviewed in 2] and toxin concentrations can vary significantly in time and space within a single bloom. Concentrations of dissolved (extracellular) microcystins are generally low during blooms since this toxin is mostly retained inside cyanobacterial cells. When a bloom collapses and cyanobacterial cells lyse, extracellular microcystin concentrations have increased to 1,800 µg/L or higher [reviewed in 2]. Following the bloom, dried crusts of intact *Microcystis* cells that washed onto shore can retain the toxin for at least 6 months [4].

Zooplankton

Microcystin exposure in zooplankton can impact feeding rate [189-191], growth [192], respiration [172], heart rate [193, 194], and survival [192, 195]. The presence of zooplankton can signal some cyanobacteria to produce more toxin [196].

In a recent meta-analysis of 66 published laboratory studies, Wilson et al. [197] found no difference between toxic and non-toxic strains of cyanobacteria in their impacts on zooplankton population growth. Such impacts, which are often reported in the literature, could be caused by poor feeding ability on, or nutrition of, the cyanobacteria as well as toxicity of lesser known cyanobacterial compounds. However, the meta-analysis showed that, in the absence of alternative food, toxic strains of cyanobacteria did impact the survival of zooplankton compared to non-toxic strains. In the majority of experiments analyzed, microcystin was the toxin present.

It is clear that zooplankton are exposed to microcystins since various species of zooplankton collected from lakes with blooms have contained significant levels of the toxin [192, 198-201]. The maximum average concentration of microcystins reported in zooplankton was 211 µg MC/g dry weight (dw) from a lake containing 12 µg MC/L (ca. 1200 µg/g dw) [199]. Individual samples from the same lake contained up to ca. 1350 µg MC/g dw [199, 201].

Key factors impacting zooplankton exposure to microcystin include the ability to avoid cyanobacteria through selective feeding (e.g., many copepods) [200, 201], cyanobacterial morphology (too big to be eaten, etc.) [197] presence of feeding inhibition responses (e.g., some *Daphnia* spp.) [202] and availability of alternative food sources [197]. Overall, zooplankton sensitivity to microcystin appears to be based on the above factors as well as species-specific sensitivities to the toxic action of microcystin [202].

Macroinvertebrates

Key factors impacting macroinvertebrate exposure to microcystins during toxic blooms are largely species-specific. Several crustaceans ingest cyanobacteria and assimilate microcystins in tissues, but not all of these are susceptible to microcystin

toxicity. Bivalve species differ in both ingestion of cyanobacteria and sensitivity to microcystin. Snails may only ingest a small portion of the cyanobacteria they consume, but are sensitive to microcystin. Like zooplankton, macroinvertebrates take up less microcystin when alternative food items are available. Regardless of sensitivities to toxicity, many macroinvertebrate species are likely to transfer microcystin to their predators.

Crustaceans

Microcystin toxicity has been demonstrated in crabs and benthic microcrustaceans. In an estuarine crab (*Chasmagnathus granulatus*), sublethal oral exposure of 5.3 µg MC/kg/day for 7 days [203] or 11.3 µg MC/kg/day for three days [204] resulted in oxidative damage (lipid peroxidation) to the hepatopancreas. Lower oral doses, 1.3 µg/kg/day for 7 days, resulted in activation of oxidative defenses, but no oxidative damage [205]. Crabs orally exposed to 172 µg/kg over three days accumulated 32 µg/kg wet weight (ww) in hepatopancreas [204]. These studies administered *Microcystis* crude extract, in which microcystin had been quantified.

The estuarine microcrustacean, *Kalliapseudes schubartii*, increased oxygen consumption following 24-h of immersion in 244 µg MC/L (*Microcystis* crude extracts) [206]. Increases in respiratory rates indicate an increased energy demand in response to the toxin, possibly due to detoxification. Longer exposure durations (i.e., 15 – 90 days) to such sublethal concentrations would further elucidate the potential impacts to individuals and populations of *K. schubartii*. The 96-hr LC_{50} of microcystin (as *Microcystis* extracts) in *K. schubartii* was 1580 µg MC/L. The 10-day LC_{50} of microcystin (as dried *Microcystis* cells) in sediment was 1945 µg MC/L.

Crayfish consume cyanobacteria but appear to be resistant to microcystin toxicity [207, 208]. Adult crayfish (*Procambarus clarkii*) fed *Microcystis* (2.3 µg MC/mg dried algae) for 2 weeks accumulated up to 2.9 µg MC/g “dry crayfish weight” [208]. Larval and juvenile crayfish (*Procambarus clarkii*) exposed to toxic *Microcystis* did not show reduced growth, nutritional status or survival. In a similar study, crayfish (*Pacifastacus lenisculus*) were fed the cyanobacterium *Planktothrix agardhii* containing 3.61 mg MC/g for 15 days. Microcystins were detected in half of the exposed animals (qualitatively) but no impacts on hemocyte counts, blood glucose levels or wet weight of hepatopancreas were observed [207].

Elevated microcystin concentrations have been measured in macroinvertebrates collected from waters with cyanobacterial blooms. Crabs collected from Septia Bay, Brazil, contained up to 0.5 µg/g dw [209]. Black tiger prawns (*Penaeus monodon*) accumulated microcystin concentrations up to ca. 80 µg/kg dw in hepatopancreas while living in an aquaculture pond with a bloom containing up to ca. 600 µg MC/g dw [210]. In a lake supporting a bloom containing up to 240 µg MC/g cells, shrimp (*Palaemon modestus*) accumulated an average of 4.3 µg MC/g dw in hepatopancreas [211].

Maternal transport of microcystin from females to eggs and young apparently takes place in crustaceans. Nearly 30% of the total microcystin body burden in shrimp

(*Palaemon modestus)* collected from a Chinese lake was found in the eggs [211]. In the same study, the gonad of crayfish (*Procambarus clarkii*) contained up to 0.93 µg MC/g dw. Dungeness crab (*Cancer magister*) larvae collected from costal Canadian waters contained up to 0.006 µg MC-LR/g dw, and much higher levels of covalently bound microcystins (up to 84 µg/g dw) [212]. The maternal transport and potential impacts of microcystin on developing crustaceans should be studied further.

Bivalves

Bivalves are generally resistant to acute lethality from microcystins [reviewed by 156]. However, recent work has indicated that these organisms are susceptible to sublethal impacts of microcystins. Zebra mussels (*Dreissena polymorpha*) fed fresh *Microcystis aeruginosa* strains with intracellular microcystin (-LR or -LF) concentrations of ca. 110 µg/L cells exhibited DNA damage in blood cells after 7 days of exposure [213]. Following 21 days of exposure, up to 30% DNA damage was observed in blood cells. Zebra mussels fed a strain with less microcystin (7 µg/L) also showed DNA damage following 21 days of exposure.

Bivalves have been found to accumulate high levels of microcystins, but the extent of accumulation appears to be modulated by both physiological and ecological factors. Mechanisms of exposure avoidance have been described in bivalves. Zebra mussels fed fresh *Microcystis aeruginosa* containing high microcystin concentrations (107 µg/L, intracellular) rejected very large quantities of the cyanobacteria as pseudofeces with copious amounts of mucus [214]. This response varied greatly from the typical expulsion of rejected particles as pseudofeces. A mixture of *M. aeruginosa* with a non-toxic diatom also produced excessive atypical pseudofeces containing significantly more of the toxic cyanobacteria compared to the non-toxic diatom. Zebra mussels fed a *Microcystis aeruginosa* strain with lower toxin concentrations (7 µg MC/L, intracellular) showed a typical response with smaller amounts of pseudofeces. Similarly, Pires et al. [215] did not observe selective feeding in zebra mussels fed a *M. aeruginosa* suspension containing 11.8 µg MC-LR/L (3.1 µg MC-LR/g dw). The long-term effects of producing excessive amounts of pseudofeces and mucus are unknown. Mucus production and the use of adductor muscles to expel the material would require increased energy. The ability of zebra mussels to expel live cells of *Microcystis* is suspected to promote *Microcystis* blooms [216, 217].

Pires et al. [215] found that the extent of microcystin-LR assimilation in zebra mussels is also dependent on the availability of an alternate food source. Zebra mussels fed a *M. aeruginosa* suspension containing 11.8 µg MC–LR/L (3.1 µg MC-LR/g dw) for three weeks rapidly accumulated a maximum of 11 µg MC/g dw. When given an equal mixture of *M. aeruginosa* and a non-toxic phytoplankton for three weeks, the mussels assimilated less microcystin (up to 3.9 µg/g dw MC-LR) at a much slower rate. In the field, zebra mussels have contained microcystin concentrations up to 30 µg/g dw [199].

Species-specific differences in microcystin uptake have also been observed. Three related bivalve species (*Anodonta woodiana, Cristaria plicata, and Unio douglasiae*) from the same hypereutrophic lake (Lake Suwa, Japan) accumulated very different levels of microcystin in hepatopancreas with maximums ranging from ca. 13 – 420 µg/g dw [218].

Intracellular toxin in surface waters reached a maximum level of ca. 35 µg/L during the study. The species with the highest accumulation, *U. douglasiae*, contained tissue microcystin concentrations correlated to the level of intracellular toxin in total suspended solids. *C. plicata* mainly accumulated microcystin following bloom collapse. The third species, *A. woodiana,* had consistently low microcystin concentrations.

Saltwater mussels (*Mytilus* spp.) can also accumulate microcystins [219-221].

Gastropods

Snails appear to be sensitive to microcystin toxicity. A common snail species (*Lymnaea stagnalis*) fed *Planktothrix agardhii* suspensions containing microcystin at 5 µg/L (280 µg/g dw) for five weeks accumulated 80 µg/g dw and showed reductions in growth (juveniles) and fecundity (adults) [222, 223]. These impacts continued after snails were fed clean food for three weeks and contained only 3.5 µg MC/g dw in their tissues. When the same species was exposed to pure microcystin-LR dissolved in experimental aquaria at a concentration of 33 µg/L for six weeks, fecundity of adults was reduced by half but no growth effects were observed in juveniles [224]. Additionally, higher levels of microcystin-LR accumulated in tissues of juveniles (7.99 ng/g ww) compared to adults (2.17 ng/g ww). The snails were apparently exposed through water ingestion rather than absorption through the skin. When up to 0.02 µg pure microcystin-LR was administered directly to the esophagus of *L. stagnalis,* histological injuries consistent with microcystin were observed in the hepatopancreas. Some of these effects were observed at relatively low microcystin-LR doses (compared to bloom exposures).

High, but naturally occurring, microcystin concentrations have been associated with lethality in snails. A mass mortality of snails was documented during a *Microcystis* bloom with microcystin concentrations up to 2500 µg/L [225]. Family richness and abundance in the macroinvertebrate community decreased as microcystin concentrations increased. Microcystin toxicity, as well as other bloom-related ecological stressors, likely contributed to the die-off.

Snails collected from waters with cyanobacterial blooms can contain high levels of microcystin. Much of the toxin can be associated with intact cyanobacterial cells in the gut and may not be digested [226]; however, these animals do digest some of the cells and take up microcystin into their bodies [227]. Three resident snail species *(Lymnaea stagnalis, Helisoma trivolvis, Physa gyrina)* from seven Canadian lakes contained microcystin-LR concentrations (up to 140 µg/g dw) that correlated with microcystin-LR concentrations in the phytoplankton, but not the water (extracellular) [228]. In a lake supporting a bloom with microcystin concentrations up to 240 µg/g dw, snails (*Bellamya aeruginosa*) accumulated average concentrations of 4.5 µg/g dw (MC-RR,-LR) in hepatopancreas [229]. Similar to many other invertebrates, gastropods are exposed to higher levels of microcystin when alternative food sources are less available [222, 228].

Fish

Fish are susceptible to sublethal toxicity from microcystins at levels commonly found in cyanobacterial blooms [reviewed by 150, 155, 156]. Uptake of microcystins by fish occurs mainly through direct ingestion of cyanobacteria or ingestion of prey that have fed on cyanobacteria [230-232]. To a much lesser extent, uptake can occur from the water [233].

Liver, kidney and to a lesser extent, gills appear to be the major targets of microcystins in fish [reviewed by 155]. Fish exposed to microcystin exhibit liver injuries including oxidative stress, cellular death (necrosis and apoptosis) and disruption of liver structure (parenchymal architecture). In the kidney, proximal tubules are most affected, showing degeneration of epithelial cells and proteinaceous casts in the lumen. Impacts on glomeruli appear to be secondary to the effects in proximal tubules. The impact on gill is less clear but cellular degeneration [234] and sodium pump inhibition [235] have been observed. Additionally, reductions in growth [236], immune status [237] and cardiac function [238] have been observed.

Reports of specific responses of fish to given doses of microcystins are sometimes contradictory. This may be partially due to incomplete purification of toxins (i.e., presence of other constituents that may affect toxicity), differences in microcystin congeners, differing experimental design (age, sex, etc.), inter- or intra-specific variation, or an incomplete understanding of factors influencing microcystin toxicity in fish. Regardless, the growing body of research on this subject provides significant insight into the threat to fish posed by *in situ* microcystin exposure.

Field observations of blooms coinciding with impacts on fish are abundant but causality of observed effects is difficult to validate. Consequently, the effects of microcystins in fish have been studied experimentally using several different fish species and exposure routes. These studies exposed fish to purified or partially purified microcystins as well as microcystin-producing cyanobacteria. Lethal extracellular (dissolved) concentrations of microcystins are unlikely to occur in the environment [reviewed by 156]. Injection studies designed to measure lethality in fish are not reviewed here since exposure by injection does not provide a useful toxicity model for natural microcystin exposures.

Immersion exposures in fish

Immersion in dissolved microcystin mimics exposures during bloom senescence when cyanobacterial cells lyse and release toxins into the surrounding waters. In this exposure, microcystins are taken up by the gills and to a lesser extent, the epidermis. In saltwater and estuarine fish, the toxin would also be taken up by drinking water, which is necessary to maintain ionic balance in a saline environment.

Brown trout exposed to lysed *M. aeruginosa* (24 - 42 µg MC-LR/L, dissolved) for 96 hours showed a clear stress response as shown by increased plasma cortisol and glucose levels and decreased plasma chloride levels [239]. Brown trout exposed to dissolved purified microcystin-LR (41 - 57 µg/L) for two months showed reduced growth

[236]. However, yearling trout placed in tanks with intact cells of *M. aeruginosa* corresponding to even greater microcystin concentrations (36.6 and 73.1 µg MC-LR/L, mostly intracellular) did exhibit effects (lethality or liver damage) within 96 hours [232]. Common carp exposed to dissolved microcystin concentrations of 1700 µg/L for seven days exhibited cellular degeneration and necrosis in liver, kidney and gills, but not in heart, spleen, or intestines [234]. These studies show that trout and carp are susceptible to sublethal effects when exposed to high extracellular (dissolved) concentrations of microcystin similar to those observed during rapid bloom senescence [reviewed in 2]. Impacts in fish exposed to dissolved microcystins during blooms include stress response, reduced growth and, at very high levels, damage to liver and kidney tissues.

Oral exposures in fish

Ingestion of cyanobacteria is considered to be the major exposure route of microcystins to fish, thus bioassays utilizing oral routes of exposure are needed [see 156]. In general, carp appear to be sensitive to sublethal microcystin toxicity following oral exposure as pure toxin or toxin within cyanobacteria. Juvenile common carp (*Cyprinus carpio*) fed a single bolus of 3 µg of microcystin per kilogram of fish body weight (denoted as µg MC/kg bw, as cyanobacteria) had elevated blood biomarkers of liver damage (as alanine transaminase; ALT) [240]. Carp given a single dose of 1200 µg MC/kg bw (as cyanobacteria) directly to the gut exhibited clear indication of damage to hepatocytes (*C. carpio*; elevated ALT, aspartate transaminase (AST) and lactate dehydrogenase (LDH)) as well as a marked change in immunological indices (*C. carpio* and *Hypophthalmichthys molitrix*) [237, 240]

Mature common carp did not show histopathological lesions in liver, kidney or gill following a single oral dose of 25 µg MC/kg bw (as *M. aeruginosa* extract). However, a single oral dose of 250 µg MC/kg caused loss of hepatic architecture and widespread necrosis in the kidney [234]. A single oral bolus of 400 µg MC/kg bw (as freeze-dried *M. aeruginosa*) in the same species resulted in damage to the kidney and liver that increased in severity over time [241]. In the kidney, vacuolation of epithelial cells, apoptosis, cell lysis, epithelial exfoliation into the tubular lumen (1 – 3 hrs post dose), proteinaceous casts (12 hrs) and ultimately disintegration of the tubular structure (24 hrs) were observed. Livers of these fish exhibited changes to structural organization of hepatocytes (1 hr post-dose), widespread damage to hepatocytes (24 hrs) and hepatocellular necrosis and apoptosis (48 hrs).

Relatively low exposure repeated over time also lead to sublethal liver damage in common carp. Repeated oral administration of microcystin (as *M. aeruginosa* extract) at dosages of 2.5 µg MC/kg bw per day for 16 days (total dose of 40 µg/kg bw) led to loss of structural architecture and widespread necrosis in liver [242]. Carp fed *Microcystis* sp. as bloom scum at a dosage of 50 µg MC/kg/day for 28 days (total dose of 1400 µg /kg bw) showed liver damage consisting of cellular dissociation and necrosis [243].

Trout are also susceptible to microcystin toxicity; however, higher doses appear necessary to cause toxic impacts in these fish. Yearling rainbow trout given single oral boluses of either 1200 µg pure MC-LR/kg bw or 1700 µg MC-LR/kg bw as crude extracts of *M. aeruginosa* did not experience liver damage as shown by histology and blood

markers [232]. However, trout given repeat oral dosages equal to 4400 µg MC/kg bw over 96 hours (eight doses of 550 µg MC/kg as crude extracts) showed a significant inflammatory response in liver with zonal leukocyte infiltration and focal necrosis [232]. Trout yearlings given a single oral bolus of *M. aeruginosa* culture at a dose of 5,700 µg MC/kg bw rapidly took-up microcystin into liver cells, resulting in widespread liver damage (loss of cellular organization, hemorrhages, necrosis and apoptosis) [231, 241]. Oral administration of 6600 µg/kg bw (as *M. aeruginosa* culture) was lethal to yearling trout within 96 hours and caused widespread lysis and degeneration of liver.

Few data are available in other fish species. Jos et al. [244] found evidence of oxidative stress (increased lipid peroxidation and induced antioxidant enzymes) in juvenile tilapia (*Oreochromis* sp.) fed ca. 1200 µg MC-LR/kg/day as *Microcystis* sp. (collected from bloom) for 21 days. Liver was most affected, followed by kidney and, to a lesser extent, gills. Perch (*Perca* sp.) given oral doses of 1150 µg MC/kg eight times over 96 hours (total dose 9200 µg MC/kg) experienced severe histopathological changes in the liver, but no mortality [199].

Exposures to sublethal concentrations could be easily encountered in typical blooms. In mature carp, oral dosages of 50 µg MC/kg/day (as *Microcystis* sp. bloom scum) for 28 days resulted in damage to liver tissues [243]. A single oral dose of 250 µg MC/kg (as *M. aeruginosa* extract) also resulted in sublethal liver damage [234]. Consider a mature common carp (5 kg) with a dry matter consumption rate equivalent to 2% of body weight (0.1 kg/day). Based on the above studies, a diet containing 2 µg MC/g dw over one month would be expected to result in sublethal effects in carp, or a species with similar sensitivity. A diet containing 12 µg MC/g could lead to sublethal effects in one day. In yearling trout, oral intake of 1100 µg MC/kg/day (as freeze-dried *M. aeruginosa*) for four days resulted in liver damage [232]. A 1-yr-old trout weighing ca. 60 g and consuming about 2 g/day (dry matter consumption at 3% of body weight) may experience sublethal liver toxicity with a short-term diet containing 35 µg MC/g dw. Such exposures are likely considering that a typical peak bloom concentration of microcystin in cyanobacterial blooms is 1,600 µg MC/g dw [2]. Bivalves, snails and zooplankton collected from areas with blooms have contained microcystins as high as 30, 140 and 1,350 µg MC/g dw respectively [199, 218]. These estimations indicate that fish residing in waters that support typical cyanobacterial blooms are likely to experience toxic effects in liver -- and some field observations support this assessment. For example, the majority of common carp sampled from a lake containing a *M. aeruginosa* bloom (2,200 – 4,000 µg MC/g bloom material dw) exhibited widespread liver damage consistent with microcystins [245]. Fish collected from a lake with recurrent cyanobacterial blooms (up to ca. 4,000 µg MC/g dw) also showed histological abnormalities in liver [199].

In summary, sublethal effects of microcystins observed in fish include progressive cellular degeneration of liver and kidney tissues involving necrosis and/or apoptosis and culminating in compromised parenchymal architecture and function. Extracts from cyanobacteria, with or without microcystins present, can also reduce growth possibly resulting from food avoidance [246] or stress response [239].

Developmental Toxicity in Fish

As mentioned above, microcystins strongly inhibit certain critical enzymes [protein phosphatase 1 and 2A 247, 248], which are necessary for most living organisms [249, 250]. These enzymes are particularly important during embryonic development in fish since their activities regulate critical developmental processes [251]. Developing fish appear to be particularly sensitive to chronic exposures to microcystins [reviewed by 155]. Observed effects include interferences with hatching, developmental defects, liver damage and reduced embryonic and larval survival. These effects appear with exposure to microcystin concentrations commonly observed during cyanobacteria blooms.

The sensitivity of developing fish to microcystins is dependent on the exposure route [252], the life stage exposed [253] and species differences [254]. Fish embryos have been shown to uptake significant levels of dissolved microcystins from the surrounding environment [186]. Increased larval mortality was observed in chub (*Leuciscus cephalus*) and zebrafish (*Danio rerio*) following embryonic exposure to 5 - 50 µg/L dissolved purified microcystins for 6 - 21 days [254, 255]. The median lethal concentration (LC_{50}) in newly hatched loach (*Misgurnus mizolepis*) larvae was 164.3 µg/L microcystin-LR (purified) for a 7-day exposure [253]. Decreased hatching rate and increased abnormalities were observed in loach embryos following exposure to 30 µg/L purified microcystin-LR for 30 days [253]. Evidence of oxidative stress was found in zebrafish embryos following a 24hr exposure to 0.25 µg/L microcystin (as purified toxin or crude extracts) [164]. Acute impacts in zebrafish eleutheroembryos (yolk-sac larvae) were only observed with very high exposures (10 mg/L) to dissolved purified microcystin-LR, and the defects (edema and enlarged yolk sac) were reversible [254].

The above effects were observed under exposures to dissolved microcystins. Experiments indicate that developing embryos would be more sensitive to exposure from maternal transport of microcystins compared to uptake from the surrounding environment [252, 256]. Maternal transfer of microcystins has been observed in shrimp collected from natural waters [211]. Microinjection of microcystin directly into developing embryos has been used to mimic potential maternal transport of this toxin. Many toxicants (especially those that concentrate in the liver) are transferred from the maternal liver to the yolk of growing eggs. Following fertilization, the embryo and larvae utilize stored yolk for the high energy demand of development [257] and are exposed to the compounds contained in the yolk.

Microinjection of 0.01 femtogram (10^{-17} g) of pure microcystin into medaka embryos significantly reduced survival [256]. Mortality increased with increasing exposures (up to 88% mortality following injection of 100 femtograms microcystin). Liver damage was observed in all medaka (*Oryzias latipes*) embryos injected with microcystin. In zebrafish, injection of similar amounts of purified microcystin-LR resulted in significant disruption of development and reduced survival [252]. Each of these microinjection studies demonstrated a dose-dependent decrease in the survival of embryos exposed to microcystins.

In summary, microcystins have been demonstrated to disrupt development in early life stages of fish. Sublethal and developmental effects are expected to occur at environmentally relevant concentrations. In general, exposure of embryos and larvae to

environmentally relevant concentrations of microcystins have resulted in evidence of oxidative stress, reduced growth, developmental defects, and lethality, as well as the lack of significant impacts. Effects occurred with a concentration as low as 0.25 µg/L or an injected dose of 0.01 femtogram/embryo. The precise mechanisms of exposure and effects in fish embryos have not been fully determined.

Birds

Bird deaths have been linked to cyanobacterial blooms in Canada and the United States since the early 1900s [reviewed by 29, 30]. Blooms of cyanobacterial species that produce microcystins and/or anatoxin-a have coincided with the deaths of ducks, gulls, songbirds, pheasants and hawks, as well as several other bird species. The severity of such bird kills have ranged from a few individuals to several thousand birds per incident.

Microcystins have been specifically implicated in some bird poisonings. In Japan, approximately 20 spot-billed ducks died at a pond containing a bloom of *M. aeruginosa* [258]. Bloom material contained high levels of microcystins and produced acute toxicity in a mouse bioassay that was consistent with microcystin. *M. aeruginosa* scum from a nearby pond contained low levels of microcystins, was not associated with bird deaths, and did not produce acute toxicity in a mouse bioassay.

In another case, waterfowl and other animals died at a reservoir containing an extensive *Microcystis* sp. bloom in South Africa [reviewed by 259]. Examined individuals showed liver damage consistent with acute and chronic microcystin toxicity. Furthermore, water from the reservoir was used to reproduce the same effects in experimental animals.

Mass mortalities of flamingos occurred in a Spanish lagoon in Donana National Park following a sudden bloom of *M. aeruginosa* and *Anabaena flos-aquae* [260]. Microcystins were detected in the water (< 10 µg/ml MC) and the crop contents (600 µg/ml) and livers (440 µg/ml) of flamingos. Both cyanobacteria species were identified in crop contents. Additionally, liver lesions consistent with microcystin toxicity were observed in the flamingos. Several other bird species also died at the lagoon.

Widespread flamingo (*Phoenicopterus minor*) mortalities have also coincided with blooms of *Arthrospira fusiformis* in alkaline lakes of Kenya [reviewed by 259, 261]. Various strains of *A. fusiformis* can produce both microcystin and anatoxin-a, which were present in flamingo carcass livers, intestine contents and fecal pellets [262]. Up to 5.82 µg/g ww anatoxin-a and 0.93 µg/g microcystins were measured in liver tissues. Additionally, neurotoxic symptoms were observed in dying flamingos [263].

In California, high mortality in birds wintering at the Salton Sea has been linked to microcystins [14]. Levels of microcystins found in many of the dead birds were similar to those in mice exposed to lethal levels of this toxin. Microcystin poisoning has also been linked to the mortality and illness of great blue heron (*Ardea herodias*) from Chesapeake Bay [14, 264].

Little experimental work has been completed in birds. Takahashi [265] reported an i.p. LD_{50} of 256 µg/kg purified microcystin RR in quail, which is low compared to that of mice [600 µg/kg, see 2]. Skocovska et al. [266] administered a daily oral dose of up to 46 µg microcystins, as *Microcystis* sp. biomass, to quail for up to 30 days. No mortality was

observed during the experiment. However, histopathological lesions were observed in livers. More work is needed to better understand the impacts of microcystins on birds.

Cylindrospermopsin

Introduction

Much less information is available on the toxic impacts of cylindrospermopsin in aquatic organisms. More knowledge on this toxin is especially needed because the geographic range of cyanobacteria that produce cylindrospermopsin appears to be increasing [reviewed by 261]. Cylindrospermopsin-producing species are now found in Australia, New Zealand, Europe, Asia and the Americas. Cylindrospermopsin is produced by *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Rhaphidiopsis curvata*, *Anabaena lapponica* and *Anabaena bergii* [reviewed by 2, 267].

The predominant route of exposure to cylindrospermopsin in aquatic organisms is through ingestion [116, 268]. As with microcystins, greater impacts are observed with exposure to crude extracts of cyanobacteria compared to purified toxins [164].

Toxic Mechanism

Cylindrospermopsin (CYN) is cytotoxic and leads to severe cellular injury and cellular death [reviewed by 151]. Past research on the toxic mechanism has focused on cylindrospermopsin's inhibition of protein synthesis, which is dose-dependent and irreversible [91]. However, other mechanisms appear to be necessary for cylindrospermopsin toxicity. When enzymes that commonly activate toxins (cytochrome P-450) are experimentally blocked, cylindrospermopsin does not lead to acute cellular death even though the impacts on protein synthesis remain the same [91, 269]. This suggests that the P-450 enzymes change cylindrospermopsin to a more toxic form, which results in cytotoxicity. The interaction of protein synthesis inhibition and the postulated cytotoxic mechanism(s) is unknown. Similarly, the key mechanisms involved in chronic toxicity are not fully understood. The main target organs of cylindrospermopsin are liver and kidney. Ingested cylindrospermopsin can also injure the cellular lining of the digestive tract. More detailed information is available in reviews by Duy et al. [151] and van Apeldoorn et al. [261].

Variants

Analog structures of cylindrospermopsin include deoxycylindrospermopsin [270] and 7-epicylindrospermopsin [271]. The toxicity of 7-epicylindrospermopsin is similar to that of cylindrospermopsin [272]. However, the toxicity of deoxycylindrospermopsin is unclear. Three mice injected with purified deoxycylindrospermopsin at a dose four-times the i.p. LD_{50} of cylindrospermopsin did not show toxic effects within five days [270]. In

contrast, purified deoxycylindrospermopsin shows similar cytotoxicity to cylindrospermopsin in several isolated cell lines [273], including hepatocytes [274]. Further study is needed to elucidate potential toxic impacts of deoxycylindrospermopsin.

Detoxification

Limited information is available on cylindrospermopsin detoxification pathways in aquatic organisms. The glutathione pathway appears to be involved to some extent [275, 276].

Blooms

Cylindrospermopsin concentrations up to 5,500 µg/g dw have been measured in bloom material [reported in 2]. Rucker et al [277] measured cylindrospermopsin in 21 German lakes dominated by *Aphanizomenon* spp. Cylindrospermopsin was detected in 19 lakes at concentrations ranging from 0.002-0.484 µg/L in seston (phytoplankton + suspended particles) and 0.08-11.75 µg/L dissolved in water. The maximum cylindrospermopsin measured in a total sample of water with seston was 12.1 µg/L. Eight of the 21 lakes sampled contained high concentrations of cylindrospermopsin. A native cyanobacterial species, *Aphanizomenon gracile*, was highly correlated with cylindrospermopsin concentrations in the lakes and was suspected to be the major producer of the toxin.

Dissolved (extracellular) cylindrospermopsin has been measured at concentrations up to 63 µg/L in natural blooms [8]. Unlike microcystin and anatoxin-a, cylindrospermopsin is often found to be highest in the extracellular fractions of surface water samples [8]. However, this is not always the case [116] and is dependent on the growth phase of the cyanobacterium [278].

Zooplankton

Cylindrospermopsin appears to reduce survival and fitness in *Daphnia*. A cylindrospermopsin-producing strain of *Cylindrospermopsis raciborskii* was more toxic to juvenile *Daphnia magna* than a similar strain that does not produce the toxin [276]. Within 48 hours, Daphnia allowed to graze on *C. raciborskii* (+CYN) experienced 90% mortality while those exposed to *C. raciborskii* (-CYN) experienced 9% mortality. Complete mortality occurred prior to first reproduction in *Daphnia* exposed to *C. raciborskii* (+CYN) (72-hrs) and *C. raciborskii* (-CYN) (192-hrs). Control Daphnia, fed a green algae, released their first brood around 192 hours and experienced no mortality within 500 hours. Effects were not solely due to poor nutritional value of *C. raciborskii* or food avoidance since starved controls only experienced 40% mortality after 500 hours of exposure. Compared to the green algae-fed controls, growth in Daphnia was reduced by 95, 80 and 30% in the *C. raciborskii* (+CYN), starved and *C. raciborskii* (-CYN) treatments, respectively, following 24 hours of exposure. Daphnia exposed to *C. raciborskii* (+CYN) contained an average 0.02 ng cylindrospermopsin per individual.

Macroinvertebrates

Crustaceans

Crayfish take up cylindrospermopsin from food and the surrounding water, but appear to be resistant to the toxin. Crayfish (*Cherax quadricarinatus*) living in an aquaculture pond with a *C. raciborskii* bloom (589 µg CYN/L, 93% within cells) and containing cylindrospermopsin concentrations of 4.3 and 0.9 µg/g dw in hepatopancreas and muscle tissues, respectively, showed no histological abnormalities in cephalothorax, digestive tract, heart, antennal gland or gills [116]. This was confirmed in experiments that exposed the same species to either a pure culture of *C. raciborskii* (128 µg CYN/L, 85% within cells) or dissolved cylindrospermopsin (as cell-free extracts; up to 568 µg CYN/L) for 14 days, which resulted in no mortalities or histological abnormalities [116]. The crayfish did consume the *C. raciborskii*, as shown by gut analyses of crayfish from the pond and culture exposure. Crayfish in the 14-day exposure to cultures of *C. raciborskii* accumulated less cylindrospermopsin than those from the pond, suggesting that time of exposure is an important factor here. In the exposure to dissolved extracts, crayfish took up cylindrospermopsin from solution at a lesser rate compared to the culture exposures.

Bivalves

The freshwater swan mussel (*Anodonta cygnea*) accumulated up to 2.9 µg/g (dw, whole body) following a 16-day feeding exposure to *C. raciborskii* containing 14 – 90 µg/L cylindrospermopsin [279]. Over 90% of the cylindrospermopsin was found in the hemolymph and viscera of the mussels. After two weeks of depuration, 50% of cylindrospermopsin remained in the tissues of the mussel. No toxicity data were found for cylindrospermopsin in bivalves.

Gastropods

Aquatic snails, *Melanoides tuberculata*, exposed to cylindrospermopsin concentrations up to 400 µg/L (either as extract solutions or live cultures of *C. raciborskii*) for 14 days showed no significant changes in behavior or relative growth rates [280]. However, the snails exposed to live *C. raciborskii* cultures at cylindrospermopsin concentrations ≥ 200 µg/L released significantly fewer hatchlings. In a similar study, snails (*M. tuberculata*) accumulated high levels of cylindrospermopsin, particularly from consumption of *C. raciborskii* cultures [268]. Higher levels of this toxin were accumulated when snails were exposed to live cultures of *C. raciborskii* compared to extracts of the cyanobacteria. Snails exposed to 25 and 400 µg/L cylindrospermopsin as extract solutions for 7 days contained 0.1 and 1.2 µg/g dw, respectively. Concentrations in these animals did not increase significantly at 14 days of exposure. In contrast, exposure to suspensions of live *C. raciborskii* cultures at a cylindrospermopsin concentration of 91 µg/L led to the accumulation of 18 and 50 µg CYN/g dw in snails over 7 and 14 days,

respectively. Snails exposed to *C. raciborskii* suspensions containing 406 µg/L accumulated up to 90 and 230 µg CYN/g dw over 7 and 14 days, respectively. The bioaccumulation factors in snails exposed to live cultures ranged from approximately 35 – 144. Deoxycylindrospermopsin was also present in *C. raciborskii* and was accumulated in a similar pattern, but at much lower tissue levels.

Fish

Rainbow fish (*Melanotaenia eachamensis*) living in an aquaculture pond with a *Cylindrospermopsis raciborskii* bloom (589 µg/L, 93% within cells) contained 1.2 µg/g dw cylindrospermopsin in viscera [116]. This accumulation was much lower than in crayfish collected from the same pond (see above). Trichomes of *C. raciborskii* were observed in the gut of crayfish, but not in Rainbow fish. Thus it appears the fish did not ingest appreciable amounts of the *C. raciborskii* in this pond. No toxicity data were found for cylindrospermopsin in fish.

Birds

No information was found on the effects of cylindrospermopsin on birds.

Amphibians

Cane toad (*Bufo marinus*) tadpoles experienced 66% mortality following exposure to live *C. raciborskii* cultures containing 232 µg/L cylindrospermopsin for 7 days [281]. In sharp contrast, no mortality occurred in tadpoles exposed to dissolved *C. raciborskii* extracts at concentrations up 400 µg/L cylindrospermopsin for 14 days. Relative growth rates and swimming activity decreased with exposure to either live culture or extracts. Tadpoles accumulated an average maximum tissue concentration of 0.9 µg/g ww cylindrospermopsin in live culture exposures and 0.06 µg/g ww when exposed to cell extracts.

Anatoxin-a

Introduction

Very limited information is available on the toxic impacts of anatoxin-a in aquatic organisms. Anatoxin-a is produced by most *Anabaena* spp. (e.g., *A. planctonica, A. flos-aquae, A. spiroides and A. circinalis*), *Aphanizomenon flos-aquae, Aphanizomenon issatschenkoi* and *Raphidiopsis mediterranea* [reviewed in 2, 261, 267]. This toxin is also produced by some species of *Planktothrix* and *Cylindrospermum*. In Japan, small amounts of anatoxin-a were produced by *Microcystis* sp. Anatoxin-a has been found in Europe, North America and to a lesser extent, Japan.

Toxic Mechanism

Anatoxin-a binds irreversibly to the acetylcholine receptors, including those that control respiration [282]. This causes overstimulation leading to paralysis and death due to asphyxiation.

Variants

Homoanatoxin-a is a homolog of anatoxin-a [283]. The two variants have very similar toxicological properties [reviewed in 261, 267]. However, anatoxin-a(s) (an analogue of anatoxin-a) is dissimilar to anatoxin-a and has a different toxicological profile [284, 285]. The toxicology of anatoxin-a(s) is not addressed here but reviews are available from Duy [151], van Apeldoorn et al., [261] and others.

Detoxification

No information was found regarding detoxification of anatoxin-a in aquatic organisms.

Blooms

Cyanobacterial bloom material has been shown to contain anatoxin-a concentrations up to 4,400 µg/g dw [286].

Zooplankton

Reproductive success in *Daphnia* was reduced when exposed to 1,000 µg/L anatoxin-a as *Anabaena affinis*, *Anabaena flos-aquae* and pure toxin [287]. These effects were exacerbated with small increases in temperatures (e.g., from 12 to 14 °C). Anatoxin-a concentrations of 200 to 5,000 µg/L (as *Anabaena flos-aquae*) inhibited reproduction in several rotifers [288]. Daphnia was more sensitive to population decline than rotifers during a natural *A. affinis* bloom but the role of anatoxin-a is unknown [289]. In copepod zooplankton, *Eurytemora affinis*, 4-day exposures to pure anatoxin-a at concentrations of 1,000 µg/L did not affect the timing or frequency of egg hatching [195]. In adult *E. affinis*, 7-day exposures at concentrations of 1,000 µg/L anatoxin-a had negligible impacts on survival. Sensitivity of a species to the overall impacts of *Anabaena* sp. may be linked to its feeding efficiency on these cyanobacteria [289, 290].

Macroinvertebrates

Crustaceans

Smith [291] suggested that sublethal cyanotoxin exposure increased the susceptibility of farmed prawns (*Penaeus* spp.) to bacterial infection and death. High prawn mortality coincided with the spread of *Oscillatoriales* blooms to new ponds, on four farms. The observed infection, vibriosis, was likely secondary to an earlier physiological insult because a number of different *Vibrionaceae* strains were present in individual prawns. Pond water was not lethal to mice, but sublethal neurotoxic symptoms were observed. Smith suggested that a neurotoxin effectively decreased the feeding and/or immune function in prawns, which led to their susceptibility to infection. This was based on neurotoxic symptoms observed in mice, lethality of pond water injected into crabs and soluble and heat-labile characteristics of the toxin.

Bivalves

No information was found regarding anatoxin-a toxicity or accumulation in bivalves.

Gastropods

Kiss et al. [292] found that specific neurons of snails (*Helix pomatia, Lymnaea stagnalisl*) responded similarly to pure anatoxin-a and acetylcholine. This work suggests that, as with vertebrates, anatoxin-a binds to acetylcholine receptors in snails.

Fish

Juvenile carp exposed to *Anabaena* sp. containing an anatoxin-a concentration of 12 µg/L exhibited behavioral changes including rapid opercular movement and abnormal swimming, but no mortality within 5 days. Carp exposed to 1,170 µg/L died within 30 hours [118]. Average whole-body concentrations of anatoxin-a after four days of exposure were 0.031 and 0.768 µg/g dw in the 12 and 1,170 µg/L exposures, respectively. In both exposures, the carp accumulated < 1% of the anatoxin-a in the experimental aquaria. The authors suspect that greater accumulation would likely occur in a medium exposure (i.e., between the lower exposure and the unknown lethal threshold), but also point out that the hydrophilicity and instability of anatoxin-a may ultimately result in insignificant accumulation in fish. Goldfish orally exposed to a *Anabaena flos-aquae* strain known to produce anatoxin-a became rigid and died within 15 minutes [111]. Immersion in fresh cells, freeze-dried cells, or extracts of *Anabaena flos-aquae* for up to eight hours did not appear to affect goldfish. Exposure to 400 µg/L, but not 200 µg/L, pure anatoxin-a temporarily altered heart rate in developing zebrafish [254]. More work is needed to understand the toxic impact of anatoxin-a on fish.

Birds

Anatoxins (including anatoxin-a) are suspected to play a significant role in observed deaths of waterfowl [156]. Mass die-offs of lesser flamingos (*P. minor*) have been related to blooms of *Arthrospira fusiformis* in alkaline lakes of Kenya [reviewed by 259, 261]. Various strains of *A. fusiformis* can produce both microcystin and anatoxin-a, which were present in flamingo carcass livers, stomach contents and fecal pellets [262]. Up to 5.82 µg/g ww anatoxin-a and 0.93 µg/g microcystins were measured in liver tissues [263]. Neurotoxic signs were observed in dying flamingos. Anatoxin-a poisoning in birds is marked by staggering, gasping, muscle fasciculation and opisthotonus [see 152, 293].

Mallard ducks orally exposed to an *Anabaena flos-aquae* strain known to produce anatoxin-a became rigid and died [111]. Several other bird deaths, including mass mortalities, have been coincident with blooms of anatoxin-a producing cyanobacteria [294].

Food Web Transfer

The movement of cyanotoxins through aquatic food webs appears to be highly site-specific. A detailed analysis of this developing field of study is beyond the scope of this report. A review of this subject is provided by Ibelings and Havens [156]. Additionally, several studies address isolated components of this topic [200, 246, 295-301].

Microcystins

The ability of microcystins to biomagnify in aquatic food webs has been debated in the literature [see 156]. However, biomagnification of this toxin is not necessary to pose a significant risk to aquatic ecosystems. What is clear is that microcystin can be taken-up into aquatic organisms faster than it is lost. This allows the toxin to move through food webs and potentially impact a greater number of species.

Studies indicate that food web transfer of microcystins are not predicted based on feeding guild (e.g., carnivores vs. planktivores) or even trophic level (primary vs. secondary consumer). Two case studies, presented below, demonstrate these limitations.

Lake Chaohu, China

Lake Chaohu is a large shallow, eutrophic lake in subtropical China. A severe *Microcystis* spp. bloom occurred from June – November, 2003. During this time various species from different trophic levels were collected from Lake Chaohu and analyzed for microcystin content [211, 229, 302]. The pelagic shrimp (*Palaemon modestus*) accumulated 4.29 and 1.17 µg MC/g dw in hepatopancreas and ovary, respectively, compared to 0.53 and 0.48 µg/g dw in the same organs of a shrimp (*Macrobrachium*

nipponensis) inhabiting the littoral zone [211]. Both shrimp species had transferred microcystin to their eggs. The pelagic species (*P. modestus*) eggs contained an average of 2.34 µg MC/g dw while *M. nipponensis* eggs held 0.27 µg/g dw.

In the same lake, crayfish (*Procambarus clarkii*) accumulated 0.93 µg MC/g dw in gonad [211]. Snails (*Bellamya aeruginosa*) collected from this lake contained average microcystin concentrations of 4.14 µg/g dw in hepatopancreas [229]. Fish species collected included carnivores (*Culter ilishaeformis, Culter erythropterus, Pseudobagrus fulvidraco, Coilia ectenes*), omnivores (*Carassius auratus, Cyprinus carpio*) a planktivore (*Hypophthalmichthys molitrix*) and a herbivore (*Parabramis pekinensis*) [302]. Microcystin accumulated in most of these fish, although concentrations varied between species. Microcystin levels were generally highest in carnivorous fish, followed by omnivorous, planktivorous and herbivorous fish. Microcystin concentrations in liver (or hepatopancreas) of species from Chaohu Lake were as follows: carnivorous fish (*Ce*) 11.6 > omnivorous fish (*Cc*) 10 > carnivorous fish (*Pf*) 7.8 > carnivorous fish (*Ci*) 5 > pelagic shrimp (*Pm*) 4.3 > snail (*Ba*) 4.1 > herbivorous fish (*Pp*) 4.1 > planktivorous (*Hm*) 2.1 > omnivorous fish (*Ca*) 1.9 > littoral shrimp (*Mn*) 0.5. In this lake, the accumulation pattern indicates a moderate accumulation of microcystin with higher trophic levels.

Lake IJsselmeer, The Netherlands

In Lake IJsselmeer, the Netherlands, a significantly different trend is observed in microcystin transfer through the food web. In 1999, lake-wide average microcystin concentration in phytoplankton was ca. 407 µg/g dw [199]. The lake-wide average microcystin concentration in zooplankton (mostly *Daphnia*) was 76 µg/g dw compared to just 6 µg/g dw in zebra mussels. In fish, average microcystin concentrations (µg/g dw) in liver tissues were highest in zooplanktivorous smelt (218), followed by benthic ruffe (54) with the predatory larger perch containing the lowest liver concentrations (24). In this lake, there was no increase in microcystin concentrations correlated with higher trophic levels.

Microcystin food web dynamics appears to be highly site-specific and dependent on local biota and food web structure. However, there are some basic principles that may facilitate site-specific assessments of the potential for microcystins to build up in certain species or groups of species. Uptake of microcystin by an aquatic species does not always lead to toxicity, which is dependent on 1) the amount of microcystin ingested, 2) lack of detoxification/excretion and 3) sensitivity to the toxic action of microcystin.

Since microcystin enters the food web through ingestion of phytoplankton (or benthic algae), the first step of the above sequence can be assessed by tracking the primary production energy flow (i.e., what organisms consume the phytoplankton). In addition to energy flow, the likelihood of grazers to actually ingest cyanobacteria must also be considered.

In Lake IJsselmeer, zebra mussels consume approximately 30 percent of the primary production while *Daphnia* spp. consume approximately 20 percent [199]. The remainder is channeled to detritus and the microbial loop. This is reflected by the microcystin accumulation in lake biota. Although zebra mussels consume a large

percentage of the lake's primary production, they are able to selectively expel cyanobacteria, which would effectively transfer microcystins to the benthos [214]. The relatively low concentrations found in zebra mussels from Lake IJsselmeer suggest that this process occurs there. Since the benthos also receives much of the lake's primary production as detritus, benthic-feeding organisms are likely exposed to microcystins. In fact, the authors found that the benthivorous fish, ruffe, accumulated significant levels of hepatic microcystin.

Unlike zebra mussels, *Daphnia* spp. do not appear to selectively reject cyanobacteria. It seems clear that the Lake IJsselmeer *Daphnia* spp. ingested cyanobacteria since the microcystin levels they contained are among the highest reported in zooplankton [156]. The zooplanktivorous smelt accumulated the highest hepatic microcystin concentrations observed in the study, apparently by ingesting *Daphnia*.

Yellow perch from Lake IJsselmeer contained relatively low levels of hepatic microcystins. Smaller perch feed on zooplankton but transition to macro-invertebrates and then small fish as they grow larger [303]. Ibelings et al. [199] stated that perch collected in the study were large enough to be predatory, but it is unclear whether macro-invertebrates or fish were dominant in their diet. Since large perch often consume smelt in this lake, it is likely that microcystin levels in this species will vary significantly with growth-dependent dietary composition.

The subtropical Lake Chaohu has greater biodiversity and a more complex food web compared to Lake IJsselmeer. The energy from primary production would be expected to move through more food web linkages than in the Dutch lake, resulting in more routes of microcystin exposure. Accordingly, the authors of the Lake Chaohu studies collected a large number of species with different feeding strategies. Species sampled from the littoral zone, which receives a high input of terrestrial food sources, contained the lowest hepatic microcystin levels. Pelagic consumers, which would likely consume a larger portion of the primary production [304], contained mid-range levels of hepatic microcystins. Pelagic predators, especially piscivores, consume primary and secondary consumers that have accumulated microcystin. This group contained the highest levels of hepatic microcystin in Lake Chaohu. An exception to the above was the phytoplanktivorous silver carp (*Hypophthalmichthys molitrix*), which would be expected to consume a large portion of the primary productivity. This fish had very high levels of microcystin in gut contents, but tissue concentrations of the toxin were low. Silver carp can have limited absorption of ingested cyanobacteria [305].

Once the species containing high microcystin levels are identified, the vulnerability of those species to microcystin toxicity should be evaluated. Toxicological studies using oral exposure routes provide relevant information on the toxic impacts of microcystin in several aquatic animals. The development of toxic thresholds for microcystin in key groups of aquatic species is needed to facilitate risk assessments of microcystin in aquatic ecosystems. Such thresholds, based on dietary or tissue burden microcystin levels, could be developed from the existing literature.

In summary, the transfer of microcystins through food webs should be assessed on a case-by-case basis. The most important factors in assessing risk of microcystin to aquatic life are exposure and toxic threshold levels. Exposure can be evaluated by monitoring microcystin levels in the dominant consumers of primary production, as well as

their predators. The scientific literature provides some information on the likelihood of some primary consumers to ingest cyanobacteria. Similarly, a large body of literature exists on the toxic impacts of microcystins to aquatic organisms. In all likelihood, wildlife management and regulatory agencies will not have sufficient resources to search the literature for species-specific exposure and effect parameters. Therefore, the development of toxic thresholds in key species is critical to site-specific risk assessment of microcystins in aquatic ecosystems.

Cylindrospermopsin

A growing body of literature indicates that cylindrospermopsin can move through aquatic food webs and accumulate in aquatic animals. Although this toxin can be taken up from surrounding water, it appears that ingestion of cyanobacteria is the predominant route of accumulation in aquatic organisms [116, 268].

Most work has focused on macro-invertebrates. Crayfish (*Cherax quadricarinatus*) living in an aquaculture pond with a *C. raciborskii* bloom (589 µg CYN/L, 93% within cells) accumulated 4.3 µg CYN/g dw in hepatopancreas [116]. However, rainbow fish (*Melanotaenia eachamensis*) living in the same pond accumulated only 1.2 µg CYN/g dw in viscera. It appears that only the crayfish had been feeding significantly on *C. raciborskii,* since the cyanobacteria was found in the gut of this species, but not in rainbow fish. Additionally, these authors demonstrated that crayfish accumulate more cylindrospermopsin from ingesting cyanobacteria than from extracellular toxin in water.

An aquatic snail, *Melanoides tuberculata*, was shown to accumulate high levels of cylindrospermopsin, particularly from consumption of cells [268]. Exposure to live cultures of *C. raciborskii* resulted in greater accumulation of cylindrospermopsin by the snails compared to dissolved extracts of *C. raciborskii*. Snails exposed to suspensions of *C. raciborskii* containing 91 or 406 µg/L cylindrospermopsin for 7 days accumulated 18 and 90 µg CYN/g dw, respectively. Accumulation increased nearly three-fold in both treatments with a 14-day exposure. In contrast, snails exposed to similar concentrations of cylindrospermopsin as extract solutions accumulated up to 1.2 µg/g dw in a 14-day exposure trial. Deoxycylindrospermopsin was also present in *C. raciborskii* and was mainly accumulated through ingestion, but at much lower tissue levels.

Cylindrospermopsin may be partially retained in macro-invertebrates following exposure. The freshwater swan mussel (*Anodonta cygnea*) accumulated up to 2.9 µg/g (dw, whole body) following a 16-day exposure to *C. raciborskii* suspensions containing 14 – 90 µg/L cylindrospermopsin [279]. After two weeks of depuration, 50% of cylindrospermopsin remained in the tissues of the mussel.

Amphibians can also take up cylindrospermopsin by ingestion of cyanobacteria. Cane toad (*Bufo marinus*) tadpoles exposed to live *C. raciborskii* cultures containing 232 µg/L cylindrospermopsin for 7 days accumulated average maximum tissue concentrations of 0.9 µg CYN/g ww [281]. Longer exposure to higher cylindrospermopsin concentrations, as dissolved crude extracts of *C. raciborskii,* resulted in the accumulation of much less toxin (0.06 µg/g ww) by the tadpoles.

More information is needed in order to understand the food-web dynamics of cylindrospermopsin. It does seem clear that ingestion is the predominant route of exposure in aquatic organisms. However, unlike microcystins, uptake of cylindrospermopsin from water can be appreciable and should be included in risk assessments.

Anatoxin-a

There is almost a complete lack of information on the potential of anatoxin-a to accumulate in aquatic organisms. Juvenile carp exposed to lethal concentrations of anatoxin-a (1,170 µg/L, as *Anabaena* sp. suspensions) accumulated average anatoxin-a concentrations of 0.768 µg/g dw (whole) prior to death (30 hrs) [118]. A much lower concentration, 12 µg/L anatoxin-a, was not lethal and resulted in average whole-body anatoxin-a concentrations of 0.031 µg/g dw. The authors speculate that greater accumulation would likely occur in a medium exposure (i.e., between the lower exposure and the unknown lethal threshold), but also point out that the hydrophilicity and instability of anatoxin-a may ultimately result in insignificant accumulation in fish.

Lesser flamingos that died during a mass mortality event had been feeding on blooms of *Arthrospira fusiformis* and contained anatoxin-a concentrations up to 5.82 µg/g ww in liver [262]. The potential for anatoxin-a to move through the food web is unknown. The chemical properties of this toxin could result in negligible transfer from prey to predator. However, more studies are needed to validate this presumption.

Conclusions and Research Needs

In conclusion, aquatic organisms residing in water bodies with recurrent cyanobacterial blooms are likely exposed to sublethal levels of cyanotoxins. The species that are exposed will depend on the toxin's movement through the food web. The sublethal toxicity of microcystins is well described. However, more work is needed on the potential impacts from maternal transport of this toxin to developing organisms. More research is needed to understand the sublethal impacts of cylindrospermopsin and, especially, anatoxin-a on aquatic organisms. The existing literature on microcystins, and perhaps cylindrospermopsin, could be used to determine sublethal toxicity thresholds in dietary items and predator tissues. This would facilitate the protection of aquatic organisms by wildlife managers and regulators. There is a strong need for an understanding of cyanotoxin's effects on aquatic mammals. Additionally, transfer of cyanotoxins to terrestrial animals deserves more attention. Several recent reviews have focused on research needs for a better understanding of the impacts of cyanotoxins on humans and animals [306-310]. Most of these reviews emphasize the need to investigate the toxicological properties of mixtures of cyanotoxins since they are most relevant to field exposures.

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